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THE REACTIONS OF CULTURE MEDIA[1]

By George C. Bunker[2] and Henry Schuber[3]

In this paper we give the results of our experience in determining the hydrogen-ion concentration of some of the commonly used media by means of color standards; we call attention to some "delightfully indefinite" statements—to use an expression of a well known chemist in a private communication—in the section of *Standard Methods* and we propose some tentative hydrogen-ion concentrations for lactose broth, nutrient agar, and Endo's agar.

The reactions of culture media in use in the laboratories of water works and the methods of adjusting the reactions are live subjects today. While the trend of opinion of bacteriologists appears to be in favor of the adjustment of the reactions of culture media by determining the hydrogen-ion concentration, yet in many laboratories, large and small, the phenolphthalein titration method is still in use. Some men continue to use the phenolphthalein titration method because they have not had the opportunity to study and work on the determination of hydrogen-ion concentrations. Others consider it inadvisable to change the reactions of media which they have used for ten or more years. Some, on the other hand, feel that a definite hydrogen-ion concentration for each medium is an unnecessary refinement. To many the term "hydrogen-ion concentration" appears too formidable because they have not seen a clear and concise article on the essential features pertaining to its application in a water works laboratory.

We venture to say that the majority of chemist and bacteriologists in water works laboratories are not members of the Society of American Bacteriologists and consequently have not seen the articles on various phases of "hydrogen-ion concentration" in the JOURNAL of this Society, the initial contribution having been made by Clark and Lubs (1917) and continued intermittently by them as well as others up to the present time; the latest article of great practical value being that of Medalia (1920). We can not expect the younger men, recently out of school, who are starting out on a modest salary, immediately to join the Water Works Associations, the Society of American Bacteriologists, the American Chemical Society, and to subscribe to the *Engineering News-Record, The American City, Engineering & Contracting,* and others. Neither can we see in the dim vista of the future many water commissioners, managers of private water companies, or corporations furnishing their laboratories with the journals of the above societies. There are probably more copies of the *Standard Methods of Water Analysis,* published by the American Public Health Association, in the hands of chemists and bacteriologists in water works laboratories than of any other book, yet in the fourth edition (1920) we find only one-third of a page devoted to the determination of the hydrogen-ion concentration of culture media. In the "Bacteriological Bibliography" of this edition there are sufficient references to the literature to enable a man located in a large city containing a large library to prime himself on the subject but for the man located in a medium sized water works laboratory there are no such library facilities. If he is sufficiently interested he must order the various journals at a considerable expense, because he can not always judge from the titles which one contains the article of greatest practical service. In this bibliography there is no reference to the excellent summary of the subject of hydrogen-ion concentration by the Committee (1919) on the descriptive chart of the Society of American Bacteriologists, which was published in the 1919 volume of the JOURNAL of this Society. In reference to a discussion of the detection of acid production of bacteria the Committee wrote as follows:

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[1] Read by title before the Chemical & Bacteriological Section, at the Cleveland Convention, June 9, 1921.

[2] Physiologist to The Panama Canal. In charge of purification of water supplies on Canal Zone and testing laboratories of Municipal Engineering Division of The Panama Canal. Ancon, Canal Zone.

[3] Chemist and Bacteriologist at Miraflores Purification Plant. Ancon, Canal Zone.

> The above discussion will be readily intelligible to any reader who has an understanding of H-ion concentration and of the meaning of the symbol pH. There would, indeed, be no point in discussing this subject further, if the research worker alone were concerned, because Clark and Lubs recent paper (1917) is readily available and treats the subject as fully and adequately as could be desired. The new chart, however, is intended primarily for instruction and it is felt that teachers may desire a brief discussion of hydrogen-ion concentration for class-room or reference use. As there is at present no bacteriological textbook, so far as the committee knows that discusses the relation of acidity and reaction of media to H-ion concentration, and as copies of this

report can be obtained from the secretary[4] of the Society at about cost price, it seems well to summarize the subject here. Nothing new, however, is added in the following pages to what previous writers have said.

While we understand that the *Standard Methods* is not intended to serve as a text book, it does appear, under the existing circumstances, as though more space should have been alloted to the subject of hydrogen-ion concentration in the fourth edition. We believe that the purchasers of copies of this edition would have welcomed the inclusion of the above mentioned summary of the Committee of the Society of American Bacteriologists even if the price of the book had been increased.

To those men who wish to determine the hydrogen-ion concentration of culture media and to adjust the reaction of the latter to definite concentrations we strongly advise the purchase of (1) Clark's book (1920) on *The Determination of Hydrogen Ions*, which contains an indexed bibliography of 1100 references; and (2) No. 5, volume 5 (September, 1920), of the *Journal of Bacteriology*, which contains an excellent article by Medalia (1920) on "Color Standards for the Colorimetric Measurement of H-ion Concentration pH 1.2 to pH 9.8." This article gives a practical method of preparing color standards for use in bacteriological laboratories and we have quoted quite extensively from it in this paper. We are not certain that single numbers of a volume of the *Journal of Bacteriology* are sold, in which case the complete volume must be purchased at a cost of $5.00 unless reprints may be secured from the author.

There is a Committee of this Association on Official Standards of Water Analysis. Is it not advisable for the Association to arrange for the cooperation of this Committee with those of the American Public Health Association, The American Chemical Society, and the referees of the Association of Official Agricultural Chemists so it may take a hand in the preparation of the next edition of the *Standard Methods*? These methods are certainly used in a sufficient number of water works laboratories to entitle a Committee of the Association to share in their preparation. We believe that the *Standard Methods* may be made more useful to the men in water works laboratories than it is at present. Moreover we doubt the wisdom of the conciseness of the description of some of the methods and subjects. It is very well to assume that "Those using these directions are thoroughly grounded in the fundamental principles of chemistry and biology, and that they are also familiar with the leading literature upon the subject," as expressed in the preface to the first edition of the *Standard Methods,* but the experience of the senior author of this paper with laboratory assistants and superintendents of purification plants, during the last eleven years, some of whom could be classed as excellent, some as good, and the remainder as poor, has convinced him that the methods should be written up in more detail to be of the greatest service to the majority of the men who use them. He also believes that there should be included pointers about certain operations to be avoided and why they should be avoided; suggestions about the preparation and standardization of various solutions; pointers about the frequency of restandardization of solutions; notations on fading of color standards, such as in the determination of iron; and so on. No matter how thoroughly a man is grounded in the fundamental principles of chemistry and biology, he will miss many points which he will pick up later by experience. Why not give the men who are starting out in water works laboratories the benefit of some of the points which have been picked up from time to time during several years experience? Why not make the *Standard Methods* a little more human, a little more interesting, and a little more helpful?

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[4] Major A. Parker Hitchens, M. C., Army Medical School, Washington, D. C.

The adjustment of the reactions of culture media to definite hydrogen-ion concentrations was adopted as a standard procedure in the Testing Laboratories of the Municipal Engineering Division of the Panama Canal in February, 1919, for reasons clearly expressed by Clark (1920):

> In the old process of adjusting the "reaction" of culture media an aliquot of a given batch was titrated to the "first faint pink" with phenolphthalein. This was supposed to give the quantity of alkali required to bring the medium to "neutrality." Then since experience had shown that a particular medium supported growth best when made more acid with a certain percentage of acid reckoned from "neutrality," the required per cent of acid, less the difference between it and the equivalent of alkali required to reach "neutrality," was added to the main batch of medium. The result of this practice was that any change in the composition of the medium changed the final pH which a given per cent of added acid would induce. In some instances the difference was enormous. Now it is generally recognized that it is not only safer and more logical but easier to adjust on a pH basis. Just as the old procedure was carried out when adjustments were made to "the neutral point of phenol phthalein" so adjustments may be carried out on the new basis with only this difference—that an indicator is chosen which brings the "zero point" at the

desired pH, as phenolphthalein brought it to the alkaline point of about pH 8.4. Having thus attained the desired reaction it is left there without the addition of a certain "per cent of acid" which we now know sent the reaction into unknown regions.

At first the following final reactions were used; for nutrient agar pH 6.7–7.0; for lactose broth, pH 6.8–7.0; and for Endo's agar, pH 7.8–8.0. Later on the final reaction of lactose broth was changed to pH 8.0. In the latter part of 1920 we went into this subject in more detail and being unable to find in the literature available to us any specific hydrogen-ion concentrations for the above media in use in other laboratories we decided to send a letter of inquiry to some of the laboratories in the United States. The information contained in nineteen answers to this letter is summarized in table 1.

REACTION OF LACTOSE BROTH

On referring to the latest (fourth) edition of *Standard Methods of Water Analysis* (1920) we find the following statement on page 95:

> *d. Sugar Broths*
>
> The reaction of sugar broths shall be a faint pink with phenol red or, if on titration with phenolphthalein the reaction is not already between neutral and +1, adjust to neutral.

On page 94 of the same book in paragraph four, under the heading bb, we find the sentence:

> The reaction of media containing sugar shall be neutral to phenolphthalein.

The above directions for adjusting the reaction of a sugar broth are conflicting in two respects and lead a man to ask:

1. Shall the reaction of a sugar broth always be made neutral as the latter of the above two quotations implies or shall it be left between neutral and +1 according to the former quotation?

2. Shall the reaction of a sugar broth be adjusted to a faint pink to P. R.[5] or to the neutral point of P.[6]

According to the first paragraph under heading bb on page 94 of the above book we are advised that the adjustment of the reaction of a medium may be made more accurate if the H. I. C.[7] is determined by means of the P. R. method yet in paragraph four under the

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[5] P. R. = phenol red.

[6] P. = phenolphthalein.

[7] H. I. C. = hydrogen-ion concentration.

TABLE I

Hydrogen-ion concentrations of media in use in various laboratories

NAME OF LABORATORY	AUTHORITY	REACTIONS OF MEDIA		
		Nutrient agar	Lactose broth	Endo's agar
Bureau of Laboratories, Department of Health, City of New York	Wm. H. Park	pH 6.4	pH 6.4-6.8	pH 8.2
State Water Survey Division, Urbana, Ill.	M. C. Perry	pH 6.6	pH 7.9	pH 6.9
Water Laboratory, State Department of Health, Albany, N. Y.	L. M. Wachter	pH 6.8	pH 7.6-8.0	pH 7.0
Iowa State College, Department of Bacteriology and Hygiene	Max Levine	pH 6.8	pH 7.0	pH 7.4
United States Department of Agriculture, Bureau of Chemistry, Microbiological Laboratory	Stewart A. Koser	pH 6.4-6.8	pH 7.2-7.4	
Treasury Department, Hygienic Laboratory, Public Health Service	M. W. McCoy	pH 7.6	pH 6.6-7.0	
Department of Hygiene and Bacteriology, The University of Chicago			pH 7.6	pH 7.6
Bacteriological Laboratory Sheffield Scientific School, Yale University	Leo F. Rettger	pH 6.8	pH 7.0	
Municipal Water Works, Springfield, Mass.	Herbert F. Salmond	+1	+.5	
Filtration Division Laboratory, Baltimore, Md.	J. R. Bayliss	"Just pink with phenol red"		
Laboratories for the State Board of Health, State University of Iowa, Iowa City, Iowa	Jack J. Hinman, Jr.	"Faint pink with phenol red"		
University of Illinois, Division of Bacteriology	F. W. Tanner	True neutrality, brom thymol blue		
University of Michigan, Hygienic Laboratory	F. E. Novy	pH 7.4 For general class work		

TABLE 1—*Continued*

NAME OF LABORATORY	AUTHORITY	REACTIONS OF MEDIA		
		Nutrient agar	Lactose broth	Endo's agar
Mt. Prospect Laboratory, Brooklyn, N. Y.	Frank E. Hale	Those given in 1912 edition of Standard Methods of Water Analysis		
State Board of Health, Vermont	B. H. Stone	Difco dehydrated media		
University of Pennsylvania, School of Public Hygiene	A. C. Abbott	pH 7.6 for all media for ordinary standard work		
Filtration Division, Bureau of Water, Pittsburg, Pa.	W. U. C. Baton	Reactions not given Adjustments to phenolphthalein titrations		
Minnesota State Board of Health, St. Paul, Minn.	H. A. Whittaker	Recently changed to adjustment by H-ion concentration. Reactions not available		
University of Missouri, Columbia, Mo.	Mazyck P. Ravenel	About to adopt adjustment by H-ion concentration		

same heading we are instructed to make the reaction of media containing sugar neutral to P. Why should we be advised to use the more accurate method of determining the H. I. C. and to adjust the reaction of a sugar broth to "a faint pink with phenol red or, if on titration with phenolphthalein the reaction is not already between neutral and +1, adjust to neutral," when on the opposite page of the book there appears the positive statement: "The reaction of media containing sugar shall be neutral to phenolphthalein."

If the more accurate method of determining the H. I. C. is to be substituted for the titration with P. and if "the reaction of media containing sugar shall be neutral to phenolphthalein," why not adjust sugar broths to a H. I. C. of 8.0, the neutral point of P. instead of 7.0 which is the point of true neutrality or the H. I. C. of pure water?

The following extract is taken from a report on the Chart for Identification of Bacterial Species by the Committee of the Society of American Bacteriologists (1918):

> It is recommended, however, that instead of using phenolphthalein in adjusting the reaction of these media the simpler and more accurate procedure be adopted of adjusting to the neutral point of brom thymol blue.[8] Bring

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[8] 0.04-per cent di bromo thymol sulphonphthalein in 95 per cent alcohol.

the media to such an acidity as to turn this indicator a distinct grass-green (neither yellow green nor blue green). This color indicates approximately "true neutrality," i.e., a hydrogen-ion concentration between pH = 6.6 and pH = 7.4, a variation which seems to have no appreciable effect on bacterial activities. Another equally satisfactory method of adjusting media to this hydrogen-ion concentration is to bring them to such an acidity as to cause the first faint trace of permanent pink to appear with phenol red.[9]

In the same report the Committee recommends that the reactions of beef extract broth beef extract agar and gelatine, and sugar broths all be adjusted to "true neutrality," i.e., to a H. I. C. between pH −6.6 and pH−7.4.

In table 1 we find that the reactions of lactose broth vary from pH 6.4 to 8.0, indicating that in some of the laboratories the reaction is adjusted to the neutral point of P., pH 8.0, in others it is adjusted to within a range of pH 6.6 to 7.4 with either P. R. or B. T. B.[10] as the indicator, and in the remainder it is adjusted to an intermediate point between pH 7.4 and 8.0. This wide range in the reaction of lactose broth is undoubtedly due to the different reactions recommended in the report of the Committee of the Society of American Bacteriologists (1918) and in the *Standard Methods of Water Analysis* (1920).

The dehydrated lactose broth prepared by the Digestive Ferments Company is adjusted to a neutral reaction and after sterilization for 20 minutes at 15 pounds pressure it is supposed to have a H. I. C. of pH 8.0. The neutral reaction must refer to neutrality to P. rather than to true neutrality.

At the present time we are preparing lactose broth according to the directions on page 95 of *Standard Methods* (1920), using Bacto-beef and Bacto-peptone. The following reactions were obtained during the preparation of a typical 3 liter batch:

Composition: distilled water (pH 6.4), 3 liters; Bacto-peptone, 15 grams; Bacto-beef, 150 grams; Bacto-lactose, 15 grams.

First reaction

To phenolphthalein............+0.8[11] To brom thymol blue pH 6.4
24.5 cc. of N/1 NaOH were then added:

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[9] 0.02-per cent phenol sulphonphthalein in 95 per cent alcohol.

[10] B. T. B. = Brom thymol blue.

[11] All reactions to phenolphthalein determined as given under "bb," page 94, "Standard Methods." (1920).

Second reaction prior to sterilization

To phenolphthalein	+0.5
To brom thymol blue	pH 7.0
To phenol red	pH 7.0

Either reaction to P. comes within the limits of "between neutral and +1," but only after the addition of the sodium hydrate does the H. I. C. come to that of "true neutrality," pH 7.0.

If the final reaction of lactose broth, prepared from the above ingredients, be changed from pH 7.0 to pH 8.0 (neutral to P.) we find that nearly twice as much sodium hydrate must be added in order to obtain the latter H. I. C., i.e., to the above batch about 47 cc. of N/1 NaOH would have been added instead of 24.5 cc.

Using Liebig's beef extract (3 grams per liter) in the place of the Bacto-beef in the formula previously given for lactose broth, the following reactions were obtained during the preparation of a typical 4 liter batch:

First reaction

To phenolphthalein	+0.7
To brom thymol blue	pH 6.4

10.5 cc. of N/1 NaOH were then added:

Second reaction, prior to sterilization

To phenolphthalein	+0.45
To brom thymol blue	pH 7.0
To phenol red	pH 7.0

While the first reaction of this batch, pH 6.4, was the same as that of the previously described typical batch prepared with Bacto-beef, we found that only 10.5 cc. of N/1 NaOH were required for the adjustment of the reaction to pH 7.0 as compared with 24.5 cc. for the Bacto-beef batch. During the preparation of 130 batches of lactose broth, using Bacto-peptone and Liebig's beef extract, we found that the average volume of N/1 NaOH required for the adjustments of the reactions to pH 6.9 to 7.0 varied from 1.5 to 2.1 cc. per liter. On substituting Bacto-beef for Liebig's beef extract but continuing to adjust the reaction to the same H. I. C. we found that the average volume of N/1 NaOH increased to 8.2 to 10 cc. per liter.

Fennel and Fisher (1919) have written as follows on the variation in peptones:

> There is a wide variation in reactions among the peptones in common use; there is even a variation between batches of the same brand.

Of several samples titrated in a 1 per cent solution, we find Witte, pH 7, Armour's pH 6.6, Difco pH 7.3, and Fairchild's pH 4.9. These samples titrated cold, with phenolphthalein as indicator, yield the following: Witte, 0.4 per cent acid, Armour, 0.7 per cent acid, Difco, 0.3 per cent acid, and Fairchild, 1.25 per cent acid.

In order to determine the effect of different brands of peptone on the H. I. C. of broth, liter batches were prepared using 5 grams each of five brands of peptone and 3 grams of Liebig's beef extract per liter of distilled water, the reaction of the latter being pH 6.4 to brom thymol blue. After the peptones had dissolved by heating to 65°C. the broths were filtered and the reactions determined both in terms of H. I. C. and the phenolphthalein titration.

TABLE 2

The reactions of beef broth with five different brands of peptone

BRAND OF PEPTONE	H. I. C. EXPRESSED IN pH	REACTION TO PHENOLPHTHALEIN 5 CC. TO 45 CC. DISTILLED WATER	
	2 cc. medium plus 8 cc. water 29°C.	Hot	Cold 29°C.
Bacto-peptone	6.6 B. T. B.	+0.7	+0.45
Witte	6.4 B. T. B.	+0.6	+0.50
Merck	5.8 M. R.	+0.85	+0.60
Fairchild	5.7 M. R.	+1.05	+0.80
Stearns	5.6 M. R.	+1.15	+0.90

Three of the above broths were found to have reactions to P., titrated hot, of less than +1, in other words their reactions ranged "between neutral and +1," so that according to the directions in "*Standard Methods*" they would be left unchanged. According to these examples and the ones previously given, it is seen that the H. I. C. of lactose broth may vary between pH 5.8 and 7.0 when the reaction falls "between neutral and +1," but if the reaction is adjusted to "neutral to phenolphthalein" it will be found that the H. I. C. will jump to pH 8.0 or 8.2. This means then that, according to the directions given in the "*Standard Methods,*" we may use lactose broth varying in H. I. C. between pH 5.8 and 8.2, depending upon the brand of peptone and whether or not we adjust the broth to "neutral to phenolphthalein." As determined at room temperature (29°C.) the reactions to phenolphthalein were all less than when determined hot and they were also "between neutral and +1."

While we always determine and adjust reactions by means of color standards we have tried the adjustment of the reaction of lactose broth by titration to "a faint pink with phenol red," with the following results:

To 5 cc. of broth added 5 cc. of distilled water and 5 drops of P. R. indicator (0.04 per cent Medalia's solution). 0.8 cc. of N/20 NaOH were required to produce a "a faint pink." Added 32 cc. of N/1 NaOH to the 4 liter batch and on again titrating obtained a "faint pink" color. With B. T. B. a pH of 6.8 was obtained. After the addition of 4 cc. more of N/1 NaOH a pH of 7.0 was obtained with B. T. B. In order to catch the development of the "faint pink" color a second dish, containing the same amounts of broth, distilled water and indicator, was used alongside the dish in which the titration was made. While we think that by using sufficient care the reaction of broth or sugar may be adjusted by this qualitative method to fall within the range of pH 6.6 to 7.4 we feel that the method is open to the same objections which have been made against the titration with P. to "a faint but distinct pink color" for the end point. In order to give a more precise description of the latter end point the use of a color top was recommended in the 1912 edition of the *Standard Methods.* A very good colored plate to illustrate the end points in the P. titration may be found on page 152 of Erye's *Bacteriological Technique,* second edition.

In some of the answers received to our letter of inquiry this qualitative method of adjusting the reaction of broth and sugar was severely criticised, for example:

> The same objection applies to the end point with phenol red as to the end point with phenolphthalein or any other indicator, that is, two observers may get entirely different results, owing to the different interpretations of the term, "a faint pink."[12]
>
> It seems to me that the directions given in the 1920 *Standard Methods for Water Analysis* for using phenol red are not good at all. The first color change of phenol red that the average worker would notice would be at least pH 7.4 or 7.6. The first noticeable color change of phenolphthalein in a medium, is at least pH 8.2 or more, depending on the worker.[13]

To correspond to the color top we have a color chart first published in Clark's (1920) book, which gives the colors of Clark and Lubs' indicators in solutions of known pH but a man must have had considerable practice with it to obtain satisfactory results.

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[12] Private communication from Stephen DeM. Gage, Chemist and Sanitary Engineer, Rhode Island State Board of Health, Providence, R. I.

[13] Private communication from Bertha van H. Anthony, M. D., Bureau of Laboratories, Dept. of Health, City of New York.

In adjusting the reaction of lactose broth by the addition of N/1 NaOH the amount first added from the titration calculation seldom brings the H. I. C. up to the desired point and it is necessary to add a few more cubic centimeters even though the exact relation between the N/20 and N/1 NaOH solutions are known. Hurwitz, Meyer, and Ostenberg (1916) recommended that a curve be plotted from a series of titrations to enable a correction to be easily made and thus eliminate a second adjustment. The reason for this difference is that in media, there are organic substances, called "buffers," which combine with some of the sodium hydroxide and thus prevent it from neutralizing the acid. Cox and Wood (1920) state:

> The presence or absence of buffering substances will have a tremendous influence on the final reaction of media adjusted according to the established method of making neutral to phenolphthalein and then adjusting to 1 per cent, 2 per cent, etc., acid on the basis of the preliminary titration. An unbuffered solution, adjusted to 1 per cent acid, would have a true reaction of very acid pH value around 5.0, while a buffered medium solution adjusted to 1 per cent acid to phenolphthalein might still be as alkaline as 7.8 ot 8.0.

According to Foster and Randall (1921):

> Broth (beef infusion, beef extract, bacto-beef) adjusted to pH values ranging from 5.0 to 9.0 undergoes a change in hydrogen-ion concentration upon autoclaving. This change is most marked in media adjusted in the alkaline range (7.8–9.0), less great in the acid range (5.0–6.2), and is usually inappreciable in the neutral range of (6.6–7.4). The maximum change is about 0.4 pH and in the majority of cases is not over 0.2 pH.
>
> The change is usually an increased acidity (decrease in pH). Decreases in acidity have been observed in a few instances but these are exceptional.
>
> Autoclaved broth undergoes changes in hydrogen-ion concentration upon standing; the degree of change is not influenced by the environmental temperature within the limits, 10°C. (ice chest) and 37°C. (incubator).
>
> The change upon standing is almost invariably in the direction of an increase in acidity.

The media prepared in the Miraflores laboratory are sterilized in an electrically heated No. 4B Bramhall and Deane autoclave of the horizontal cylindrical type, 27½ inches long by 24½ inches in diameter, inside measurements. With this type of autoclave and holding the broth in flasks at 17 pounds pressure for 20 minutes, the total lapse of time from the insertion of the medium to its removal averages 1 hour. In the Mt. Hope and Agua Clara laboratories there are used electrically heated autoclaves of the vertical cylindrical type, 24 inches high by 11 inches in diameter, inside measurements. With this type of autoclave and holding the broth in flasks at 17 pounds pressure for 20 minutes, the total lapse of time from the insertion of the medium to its removal averages 60–80 minutes. There is an additional sterilization for 80 minutes at 100°C. after the addition of the sterile lactose solution and the tubing of the broth. The reactions of batches of lactose broth, adjusted to either pH 6.9, 7.0, or 7.1 before sterilization were not appreciably changed by the autoclaving.

In reference to the change in lactose broth stored in an ordinary large size refrigerator of the house type we have found at the end of seven days that the H. I. C. has changed from pH 7.0 to 6.8 or 6.9. In the Miraflores laboratory lactose broth is made up in 4 liter batches at intervals of three or four days so it is seldom that tubes of it are stored in the refrigerator for more than four days at the end of which time no appreciable change in the H. I. C. has taken place. If broth is to be stored in the refrigerator of a laboratory for more than seven days allowance should be made for a change in the H. I. C.

We have found that the final H-ion concentrations of several tubes of lactose broth filled from the same batch always agree very closely.

Wagner and Monfort (1921) in their discussion on lactose broth raise the following point:

> The reaction, adjusted to neutrality prior to autoclaving, is greatly modified by the alkalinity of natural waters tested; the pH values of 6.6 to 7.4 rarely prevail in fermentation tubes as incubated.

With the waters available to us, ranging in pH values from 4.9 to 8.1, we have not found this to be the case for the pH values of waters mixed with the broth in 1 and 10 cc. fermentation tubes have neither fallen below pH 6.8 nor exceeded pH 7.0. 10 cc. and 25 cc. of broth, respectively, were used in the Durham fermentation tubes to which 1 cc. and 10 cc. of water are added. The concentration of lactose in the mixture of 10 cc. of water and 25 cc. of broth was 0.33 per cent, while in the mixture of 1 cc. of water and 10 cc. of broth it was 0.42 per cent. Table 3 contains the data relating to this point.

In determining the H. I. C. of lactose broth by means of P. R. and the standards put up by Hynson, Westcott, and Dunning, 10 cc. of the broth is placed in a test tube and 1 cc. of a 0.01 per cent aqueous solution of the indicator added. If the indicator is added to the warm broth and the color compared with the above standards we find that the warm broth throws the color of the mix-

TABLE 3

The change in hydrogen-ion concentration of lactose broth by the addition of waters of various alkalinities

KIND OF WATER	ALKALINITY, PARTS PER MILLION		HYDROGEN-ION CONCENTRATIONS EXPRESSED IN pH		
	Erythrosine	Carbonate (CO_3)	Water	Lactose broth plus	
				1 cc. water	10 cc. water
Chagres River, raw water supply for Miraflores Purification Plant	54.1		7.2	6.9	7.0
Agua Clara reservoir, raw water supply for Agua Clara Purification Plant	25.4		6.9	6.9	6.9
Settled water from above Plant	1.0		4.9*	6.8	6.9
Filtered water from above Plant.	20.4		7.3	6.9	6.9
Rio Grande reservoir	64.3	4.8	7.6	6.9	6.9
Ground water, Corozal road, Canal Zone	113.2		7.5	6.9	6.9
Sea water in swimming pool, Colon, R. de P.	132.6		7.7†	7.0	6.9
Santa Ana spring water, Costa Rica	377.6	26.4	7.6	7.0	7.0
Well water, Barranquilla, Colombia	502.1	68.5	8.1†	7.0	7.0
Ditto	167.3	14.4	7.8†	7.0	6.9

Indicators: * = methyl red; † = phenol red; all other determinations, brom thymol blue. The pH value of the lactose broth in all the tubes was 6.9. All hydrogen-ion concentrations were determined by diluting 2 cc. of the contents of the broth tube with 8 cc. of distilled water with a pH value of 6.4.

ture off shade a little. Therefore when determining the H. I. C. of broth in this manner we recommend that it be cooled to room temperature before the P. R. is added. In determining the H. I. C. by means of B. T. B. and the standards prepared after Medalia, 2 cc. of the broth are added to 8 cc. of distilled water which has been kept at room temperature, i.e., about 29°C., and then 0.8 cc. of the indicator. The shade of this mixture compares very closely with that of the standard color, the 8 cc. of distilled water serving to cool the broth.

The question is apt to arise in the mind of a man who has not had the opportunity to go into all the details of hydrogen-ion work that an error is introduced by the dilution of 2 cc. of lactose broth or nutrient agar with 8 cc. of distilled water which has a H. I. C. different from that of the former. In reference to this point Foster and Randall (1921) have written:

> Preliminary tests showed that with broth and cultures it was possible to dilute 1 cc. of the material with 4 cc. of distilled water without altering the hydrogen-ion concentration.
>
> Freshly boiled and cooled distilled water was used for diluting as preliminary tests had shown that unboiled water gave slightly lower pH readings. The pH of the water itself was usually found to rise from 4.8 to 6.8 upon boiling, probably due to the liberation of carbon dioxide.

The distilled water used in the Miraflores laboratory is produced by a Barnstead automatic type E still, electrically heated, and as removed from the storage bottle has an average pH of 6.4. With lactose broth and nutrient agar we have determined the H. I. C. by adding in one case 2 cc. of the medium to 8 cc. of distilled water and in another case by using 10 cc. of the undiluted medium and comparing the colors with the standards prepared after Medalia. No appreciable difference was found in the hydrogen-ion concentrations of the diluted and undiluted media, the reaction of which had been adjusted to pH 6.9 to 7.2.

Figure 1 after chart 8 in the paper by Cox and Wood (1920):

> illustrates the dissociation of the hydrogen-ions in a medium at varying temperatures. The plain extract broth medium used here has a pH of 8.4 at room temperature (20°C.) and a pH of 7.4 at 70°C., and the varying pH values indicated at the intervening temperatures. It is necessary to adjust titrations so that the end point will be the pH desired at the temperature at which the medium will actually be used. This can be done by the use of a constant temperature bath and by adjusting the reaction of the medium in preparation

at exactly the required temperature. It must be remembered that pH 7.0 is the true neutral point only at room temperature (20°C.), variations in temperature from this value alter our total pH scale, hence it is essential to determine and include, in our definitions of optimum pH values, the temperature.

From the above we judge that in adjusting the reaction of a batch of lactose broth we should bring it to a temperature of 37.5°C., at which it will be incubated later on when in use, before determining the H. I. C. and adding the correct amount of N/1 NaOH. The 2 cc. portions of broth removed from the batch should also be diluted with distilled water of a temperature of 37.5°C. In general the temperature of a batch of broth in the Miraflores laboratory at the time of the addition of normal alkali to bring the reaction to pH 7.0 averages 31.5°C. while the temperature of the mixture of 2 cc. of broth, 8 cc. of distilled water and 0.8 cc. of indicator averages 29.5°C. On incubating tubes of broth adjusted to pH 7.0 under these conditions at 37.5°C. for 24 hours we found that the H. I. C. was increased to 7.2 and remained at this point after a second period of 24 hours or a total of 48 hours. It appears, therefore, that the broth should have been originally adjusted to pH 6.8 in order to insure a H. I. C. of 7.0 during the early part of its incubation

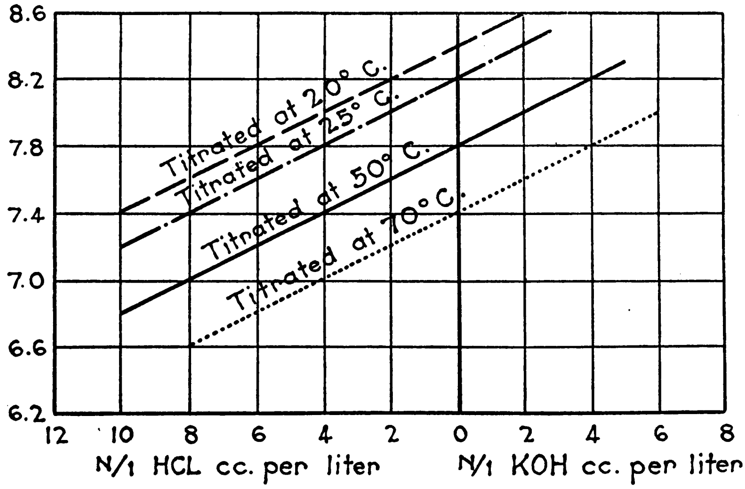

FIG. 1. COMPARATIVE TITRATIONS OF THE SAME MEDIA AT DIFFERENT TEMPERATURES

but as the addition of waters of different H-ion concentrations introduces another variable factor it is hardly worth while to make a correction for the difference in temperature at the time of adjustment of the reaction or to make use of a constant temperature bath.

For keeping a permanent record of the composition and reactions of the batches of lactose broth prepared in the laboratory we employ a note book measuring 6 inches long by 3¾ inches wide. On the inside of the front cover there is pasted the typewritten directions for the preparation of the broth which are followed by the laboratory helper until they are superseded by a new set of directions in case of changes in the composition of the broth. On the first page of the book facing the inside of the front cover there is pasted a second typewritten sheet containing the various entries to be made by the laboratory helper on the blank pages of the book before the batches of broth are prepared. Opposite some of these entries the bacteriologist records the reactions and at the bottom of the pages signs his initials to indicate that the composition, preparation and final reaction of the broth are correct. Table 4 is a copy of a page in this note book.

TABLE 4

Specimen page of lactose broth record book

Batch 9. Lactose broth. 4/30/21

	grams
Weight of can	1500
Weight of distilled water	4000
Weight of bacto-beef	200
Weight of bacto-peptone	20
Total	5700

40 cc. N/1 NaOH added

Reaction, pH 7.0 with B. T. B.

Reaction before sterilization	pH 7.0 B. T. B.
Reaction after sterilization	pH 7.0 B. T. B.
Weight of bacto-lactose	20 grams

Chemists O.K., H. S.

On the inside of the cover of the daily note book, in which the bacteriological analyses of the daily samples of water are recorded, there is pasted a sheet on which entries are made to show between what dates the various batches of lactose broth are used. There is also pasted on the inside of this cover a sheet which shows the composition and the method of preparation of the broth used during the period covered by the notebook. If a change is made in the composition of the broth during this period the changed formula is entered on this same sheet. In this manner a permanent record is kept which eliminates the vagaries of the memory of the man in charge of the laboratory and which enables a man going over the notebooks a few months or years later to obtain definite information concerning the broth used during any period.

In conclusion it appears desirable to decide whether lactose broth is to be adjusted to "neutral to phenolphthalein" or to "true neutrality," thus eliminating the wide range of H. I. C. resulting from the use of a reaction "between neutral and +1." If the reaction is adjusted qualitatively, that is, by means of a certain color tint of an indicator, we may expect to have the following ranges in H. I. C.:

Brom thymol blue or phenol red	pH 6.6 to 7.6
Phenolphthalein	pH 8.0 to 9.0

We recommend that the reaction be adjusted by means of color standards, prepared either with B. T. B. or P. R. as the indicator, and that the range of H. I. C. be limited to 6.8 to 7.2. We also recommend the same range of reaction for nutrient broth.

REACTION OF NUTRIENT AGAR

On referring to the latest edition of *Standard Methods* (1920) we find the following statement on page 96:

> *f. Nutrient agar*
>
> 3. Make up lost weight and adjust the reaction to a faint pink with phenol red, or if the phenolphthalein titration is used, and the reaction is not already between +0.5 and +1, adjust to +1.

In the *Standard Methods for the Bacteriological Examination of Milk* (1921) we find on page 8 the following statement under the heading of "Reaction":

> Inasmuch as the range of H-ion concentration recommended for water analysis is pH = 6.8 to 8.4, it is permissible, if desired, to use a single agar for both purposes (milk and water analyses—authors' insert) with a reaction of pH = 6.8 to 7.0. If Witte's peptone is used in the above formula, this will ordinarily be the reaction without adjustment.

What is meant by the clause, "Inasmuch as the range of H-ion concentration recommended for water analysis is pH 6.8 to 8.4?" Does the Committee mean that for water analysis nutrient agar may be adjusted to a reaction varying between these limits? While this interpretation does not appear reasonable on second thought, yet it is entirely plausible for *agar* is the only medium under discussion. A reference in the above paragraph refers to page 93 of *Standard Methods*, a. Adjustment of Reaction.

> aa. Phenol red method for adjustment to a hydrogen-ion concentration of pH = 6.8–8.4.

This leads us to inquire why this range, when on the following pages the only reaction referred to in the adjustment of the media is that of faint pink with P. R. As previously quoted the Committee of the Society of American Bacteriologists recommends that the reaction of beef extract agar be adjusted to the neutral point of B. T. B. or to the first faint trace of permanent pink with P. R., which colors indicate approximately "true neutrality," i.e., a H. I. C. between pH, 6.6 to 7.4. If the range of pH 6.8 to 8.4 in the *Standard Methods* for both milk and water analysis is a misprint for 6.8 to 7.4 and the latter is substituted in the first sentence of the paragraph quoted from the *Standard Methods* of milk analysis, the meaning of the paragraph becomes clearer. If it is not a misprint we believe that pH 8.4 should be changed to pH 7.4, because all of the reactions adjusted to a faint pink with P. R. would come within the range of pH to 7.4 if carefully taken.

Perhaps the range of pH 6.8 to 8.4 was used because this covers the entire range of P. R. If so, did the Committee think that one could not come closer to a "faint pink" than the red of pH 8.4 or the yellow shade of pH 6.6? If so what is the improvement over the indefinite end point of the phenolphthalein titration?

Whatever may be the correct interpretation of the sentence under discussion the Committee on the *Standard Methods* of milk analysis has made it clear that nutrient agar with a H. I. C. of 6.8 to 7.0 is suitable for both milk and water analysis. The Committee of the Society of American Bacteriologists recommends that the reaction of beef extract agar be adjusted to "true neutrality," so that for this medium the two Committees are in close agreement.

In table 1 we find that, while the reactions of nutrient agar vary from pH 6.4 to 7.6, the majority of the laboratories keep within the limits of pH 6.4 to 6.8, slightly lower than the range recommended above. The dehydrated nutrient agar prepared by the Digestive Ferments Company is adjusted to a reaction between +0.5 and +1.0 and after sterilization for 20 minutes at 15 lbs. pressure it is supposed to have a H. I. C. of 6.5.

At the present time we are preparing nutrient agar according to the directions on page 96 of *Standard Methods* (1920). The following reactions were obtained during the preparation of a typical 3 liter batch: Composition; grams; distilled water (pH 6.4), 3000; dried bacto-agar, 36; bacto-peptone, 15; Liebig's beef extract, 9.

First reaction

To phenolphthalein	+0.4
To brom thymol blue	pH 6.4

7 cc. of N/1 NaOH were then added.

Second reaction

To phenolphthalein	+0.3
To brom thymol blue	pH 6.9

1 cc. of N/1 NaOH was then added.

Third reaction

To phenolphthalein	+0.3
To brom thymol blue	pH 7.0
To phenol red	pH 7.0

The final H. I. C. of the agar is that of "true neutrality" while the reaction to phenolphthalein is +0.3. According to the directions in *Standard Methods* (1920) for adjusting the reaction with the latter indicator, the addition of N/1 HCl is necessary in order to bring the reaction to +1, because the first reaction was not between +0.5 and +1. In order to show what happens under such conditions we adjusted the reaction of a batch of agar with an initial reaction of +0.4 to P. to a final reaction of +1, which brought the H. I. C. to 4.9 with M. R.

Plates were made from this agar and compared with some made from the other portion of the batch which was adjusted to pH 7.0. The numbers of colonies which developed on the kinds of agar during 24 hours incubation at 37.5°C. were as follows:

WATER	COLONIES ON AGAR	
	pH 7.0	pH 4.9
Raw	300	4
Raw	250	2
Aerated	350	2
Aerated	375	8
Settled	230	2
Settled	250	2
Settled	375	6
Settled	190	2

It is seen that the more acid agar inhibited the growth of the bacteria. It would have been preferable, at least in our laboratory, to have left the reaction of this batch of agar at +0.4 to P. or at pH 6.4 instead of changing it to +1. The appearance of the tubed pH 4.9 agar after two days was hard but on inverting and shaking a tube the agar was found to be slushy. Another batch of nutrient agar, final reaction pH 4.9, after storage in the refrigerator for 14 days, was so slushy that plates could not be made from it.

The reactions of 24 batches of nutrient agar, prepared prior to the date on which we started to take the H. I. C., averaged +0.5 to P., using the end point of the color top previously described under lactose broth. Under this condition it was thought best to leave the reaction alone and not add any acid to the agar. After adjustment of the reaction according to the H. I. C. was started, the use of the same materials being continued, it was found necessary to add 2 cc. of N/1 NaOH per liter of agar to obtain a final pH 6.9 to 7.0. In general it may be said that the H. I. C. of the agar with a final reaction of +0.5 to P. was pH 6.6 so that when we changed the final reaction of this medium to pH 6.9 or 7.0 we did not notice any difference in the counts. At the present time we find that the first or "native" reaction of nutrient agar, of the composition previously given, averages pH 6.4 to 6.6 and that in general 2.5 to 3 cc. of N/1 NaOH per liter must be added to give a final H. I. C. of 6.9 to 7.1 with B. T. B.

One cannot be certain that a reaction to P. will always have a corresponding fixed H. I. C. equivalent. For example we have found at times that a batch of agar with a reaction of +0.3 would have a pH of 6.9 or 7.0; again that a reaction of +0.5 would correspond to a pH of 6.6 or less (at this time we had no standard lower than 6.6); and at other times that a reaction of +0.6 would correspond to a pH of 6.9.

Fennel and Fisher (1919) state:

> There is no constant interrelationship between pH readings and phenolphthalein titrations, except in a case where a definite relationship has been established, in a medium of absolutely standard construction, using distilled water, the same batch of meat extract and peptone. Any change in technic changes the relationship between the two systems of titration; as a matter of fact, the pH is so satisfactory and practical, that this relationship is only of academic interest.

While we always determine and adjust the reaction of nutrient agar by means of color standards we have tried the adjustment of the reaction by titration with B. T. B. to a "distinct grass green," with the following results:

To 5 cc. of distilled water added 5 cc. of the agar and 5 drops of B. T. B. (0.02 per cent) and titrated in a casserole with N/20 NaOH. According to this titration we added 8 cc. of N/1 NaOH to the 4 liter batch and obtained a reaction of pH 6.8. The same objections apply to this qualitative method of adjusting the reaction of nutrient agar as were given under lactose broth. A further addition of 4.3 cc. of N/7 NaOH was made to the above batch in order to obtain a final pH 6.9.

The nutrient agar prepared in the Miraflores laboratory for the laboratories of the three purification plants is sterilized in the large autoclave described under lactose broth and is held in it for an average period of one hour from the start to the finish of the sterilization. The reactions of batches of nutrient agar, adjusted to either pH 6.9, 7.0 or 7.1 before sterilization were not appreciably changed by the autoclaving.

Tubes of nutrient agar, pH 6.9 to 7.1, did not show any appreciable change in H. I. C. when stored for 15 days in the same refrigerator mentioned under lactose broth. In the Miraflores laboratory nutrient agar is made up in 4 liter batches at intervals of 4 to 5 days so it is seldom that tubes of it are stored in the refrigerator for more than 4 days at the end of which time no appreciable change in the H. I. C. has taken place. The nutrient agar for the Mt. Hope and Agua Clara laboratories is prepared in the Miraflores laboratory and shipped to them in tubes ready for use. All the agar is prepared under the supervision of the same bacteriologist and the reaction of each batch is adjusted to the same H. I. C. in order to eliminate the variations which would creep in if it were prepared in three different laboratories.

Fennel and Fisher (1919) make the statement that:

> Agar mediums are best adjusted in the broth stage, and the agar added as the last step. Agar is, for all practical purposes, neutral in reaction, and does not change the pH of the broth. If this should not be convenient, the agar may be melted and titrated hot. In comparing the colors with agar it is best to wait until the agar has set before concluding that the colors are identical. It is a simple matter to send this tubed specimen, with indicator in it, along with the batch of mediums, through the sterilization process to check the reaction after sterilization.

In reference to the last sentence in this quotation we have tried sending two tubes of nutrient agar through the autoclave, 10 cc. each, to one of which 0.8 cc. of 0.04 per cent P. R. had been added and to the other of which 0.8 cc. of 0.02 per cent B. T. B. had been added. The tube of agar containing the P. R. gave a reaction of pH 7.1 and that containing the B. T. B. gave a reaction of pH 6.9 with the latter indicator. According to this experiment we believe it best to obtain the final reaction on tubes of agar which have gone through the autoclave without any added indicator.

We have experienced no difficulties in adjusting the reaction of broth in which agar has been dissolved and as the final reaction must be taken on the nutrient agar ready for use we think it best to make all the H-ion determinations on the broth containing the agar. We like the color shades produced by B. T. B. better than those produced by P. R. when color standards prepared after Medalia are used in the comparator block which will be described in a later section of this paper. In this connection Medalia (1920) observed that:

> The phenol red does not make as clear cut a range, as easily differentiated at pH 0.2 intervals, as does brom thymol blue and is not really necessary for the titration of culture media which as a rule are neutral, or thereabouts.

We also prefer to determine the H. I. C. of nutrient agar by means of color standards prepared after Medalia rather than by means of the set of color standards prepared by Hynson, Westcott and Dunning as we believe that the color of the unknown can be matched more closely with that of the standards.

In determining the H. I. C. of nutrient agar by means of B. T. B. and standards prepared after Medalia 2 cc. of the agar are added to 8 cc. of distilled water, which has been kept at room temperature, i.e., about 29.5°C., and then 0.8 cc. of the indicator, 0.02 per cent solution. The color of the nutrient agar tube may then be matched immediately against the standards or allowed to stand five or ten minutes, for the dilute agar will not solidify, but we have observed that if allowed to stand for 20 minutes or over the color of the unknown will grow slightly darker. In the *Standard Methods for the Bacteriological Examination of Milk* (1921), the use of distilled water of a temperature of 30 to 40°C. is advised but we do not see the necessity of using water of this temperature and believe it is preferable to have the temperatures of the unknown and the standards about the same, that is, at the temperature of the room in which the latter are kept.

In determining the H. I. C. of nutrient agar by means of P. R. and the standards put up by Hynson, Westcott and Dunning 10 cc. of agar is placed in a test tube and 1 cc. of the indicator added (0.01 per cent aqueous solution). In this case the agar should be cooled to room temperature before the colors are compared as we have observed that the pH 7.0 of a tube of hot agar increased to pH 7.1 on cooling. Hurwitz, Meyer, and Ostenberg (1916) advise that:

> Readings should be made on the solidified medium at a constant room temperature, since the hydrogen-ion concentration changes with the temperature.

A permanent record of the composition and reactions of the batches of nutrient agar prepared in the Miraflores laboratory is kept in the same manner as described under lactose broth in a separate note book. Table 5 is a copy of a page of this book.

TABLE 5

Specimen page of nutrient Agar Record Book

Batch 4. Nutrient agar. 4/16/21

	grams
Weight of can	1182
Weight of distilled water	4000
Weight of bacto-agar	48
Weight of bacto-peptone	20
Weight of beef extract	12
Total	5262

10 cc. N/1 NaOH added.
Reaction, pH 7.0 with B. T. B.
Reaction, before sterilization.........................pH 7.0 with B. T. B.
Reaction, after sterilization..........................pH 7.0 with B. T. B.

Number of tubes shipped to Gatun..........................120 on 4/18/21
Number of tubes shipped to Mt. Hope.......................140 on 4/18/21
Chemist's O. K. H. S.
Colonies on trial plates, 0-0-0-0-0

In conclusion it appears desirable to eliminate the adjustment of the reaction of nutrient agar by titration with P. and to substitute for the latter either B. T. B. or P. R. If the reaction is adjusted qualitatively by means of either of the last two indicators we may expect to have a range in H. I. C. of pH 6.6 to 7.4. Inasmuch as the Committee on the *Standard Methods* of milk analysis recommended a reaction of pH 6.8 to 7.0 and the Committee of the Society of

American Bacteriologists recommended a reaction of true neutrality we suggest that the reaction be adjusted by color standards, prepared either with B. T. B. or P. R. as the indicator, and that the range of H. I. C. be set at pH 6.8 to 7.2, the same as suggested for lactose broth. We also recommend the same range of reaction for nutrient gelatin.

REACTION OF ENDO'S AGAR

Composition, grams; distilled water, 1000; Bacto-agar, 30; Bacto-peptone, 10; Liebig's beef extract, 5.

On page 97 of the *Standard Methods* (1920) under "1. Endo's Medium," the reaction of this medium is specified as follows:

> 3. Make up lost weight, titrate, and if the reaction is not already between neutral and +1 adjust to neutral.

During 1920 the reaction of batches of Endo's agar at the above step in its preparation varied from +1.5 to 2.2 and from 18 to 23 cc. of N/1 Na_2CO_3 were required per liter to bring the reaction to neutral to P. We did not start to adjust the reaction of this medium to pH 8.0 to 8.2 until the early part of the current year so we have no H-ion concentrations to correspond to the above acidities to P. At the present time we find that the reaction to P., prior to adjustment, averages 1.7 and that 12.5 cc. of N/1 Na_2CO_3 per liter are required to give a pH of 7.2 which is increased to a final pH of 8.0 by the addition of the chemicals. A recently prepared batch of agar gave the following reactions:

First reaction

To phenolphthalein	+1.7
To brom thymol blue	pH 6.4

12.5 cc. of N/1 Na_2CO_3 per liter added:

Second reaction

To phenolphthalein	+0.5
To brom thymol blue	pH 7.2

A portion of this batch was adjusted to a reaction of neutral to P. according to the above quotation and the H. I. C. found to be 8.0 to P. R. which would be increased to a final H. I. C. of 8.8 after the addition of the chemicals. The colonies growing on plates prepared from this batch were unsatisfactory as compared to those developing on the remainder of the original batch which had a final reaction of pH 8.0. Five liters of agar were prepared according to the formula given at the head of this section and divided into five portions of 1 liter each, to which various amounts of N/1 Na_2CO_3 were added in order to obtain different H-ion concentrations. The five batches were sterilized in the autoclave used for the lactose broth and nutrient agar at 17 lbs. pressure for 20 minutes after this pressure was reached.

To 250 cc. portions of the different batches of this medium, the following amounts of chemicals were added immediately prior to the preparation of the plates:

> 1.25 grams of Merck's sodium sulphite in 25 cc. of water. 2.5 cc. of a 10 per cent alcoholic solution of basic fuchsin (Harmer Laboratories). In making this solution the fuchsin is allowed to stand in contact with the alcohol for 24 hours at the end of which time it is filtered.
>
> 2.5 grams of Bacto-lactose in 30 cc. of water.

TABLE 6

Hydrogen-ion concentrations of five batches of Endo's agar, expressed in pH

BATCH NUMBER	N/1 Na_2CO_3 ADDED	HYDROGEN-ION CONCENTRATIONS, pH		
		Before autoclaving	After autoclaving	Final as poured into plates
	cc.			
1	8.0	7.2	7.2	8.0
2	8.0	7.2	7.2	8.2
3	6.0	7.0	7.0	7.8
4	3.0	6.6	6.6	7.4
5	None	6.4*	6.4*	7.2

Chemicals added to Batch 2 prior to autoclaving.

H-ion concentrations marked * were taken with B. T. B., using standards prepared after Medalia; the others were taken with a set of standards prepared by Hynson, Westcott and Dunning, with P. R. as the indicator.

The sodium sulphite, into which the fuchsin had been poured, was added to the solution of lactose and the resulting mixture poured into the agar. The H-ion concentrations of the five batches at different stages are shown in table 6.

Plates of the five different batches of agar were prepared from tubes of lactose broth inoculated with human faeces and different waters, and compared after 24 hours incubation at 37.5°C. in order to see which batch produced the most distinctive appearing colonies. Three observers judged the colonies on each plate and the summary of their observations is given in table 7.

These tests were repeated several times and we could not detect much difference in the appearance of the colonies growing on batches 1, 2, and 3. In general we thought that better colonies were obtained on batch 1, with those on batch 2 running a close second. The colonies on batch 3 did not carry quite as much metallic sheen and had a tendency to be irregular. Batches 4 and 5 were clearly unsatisfactory, as the growth and sheen were always very light. As a result of these tests we decided to adopt a final reaction of pH 8.0 to 8.2.

TABLE 7

Appearance of colonies on Endo's agar after 24 hours incubation at 37.5°C.

BATCH NUMBER	APPEARANCE OF COLONIES ON PLATES INOCULATED WITH A LACTOSE BROTH CULTURE OF HUMAN FAECES
1. pH 8.0	Distinct, typical, with metallic sheen and halo. Agar colored pink. Colonies called very good
2. pH 8.2	Similar to those on batch 1. Agar colored pink. Colonies called very good
3. pH 7.8	Similar to those on batch 1 and 2 but not as good. Agar colored red
4. pH 7.4	Unsatisfactory
5. pH 7.2	Unsatisfactory
BATCH NUMBER	**APPEARANCE OF COLONIES ON PLATES INOCULATED WITN LACTOSE BROTH CULTURES OF DIFFERENT WATERS**
1. pH 8.0	Distinct, typical, with metallic sheen and faint halo. Agar colored red
2. pH 8.2	Distinct, typical, with metallic sheen. Halo more pronounced than on batch 1 but not strong. Agar colored pink
3. pH 7.8	Distinct, typical, with metallic sheen. Halo of about same distinctness as on batch 2. Agar colored red
4. pH 7.4	Unsatisfactory
5. pH 7.2	Unsatisfactory

Dr. Hans Zinsser, College of Physicians and Surgeons, Columbia University, Dr. Leo F. Rettger, Sheffield Scientific School, Yale University, and Dr. Wm. H. Park, Bureau of Laboratories, Department of Health, City of New York, have advised in private communications a final reaction of pH 8.0 to 8.2 for Endo's agar. Fennel and Fisher (1919) and Kliger (1918) also recommend a H. I. C.

of 7.8 to 8.0 as the optimum reaction for Endo's agar. The dehydrated Endo's agar prepared by the Digestive Ferments Company is stated to have a reaction of pH = 7.0. when sterilized for 20 minutes at 15 pounds pressure. In this connection we must call attention to a mistake which we made in our letter of inquiry, sent out in January, 1921, in giving the reaction of Endo's agar as pH 7.0 and not stating that this was the reaction prior to the addition of the sodium sulphite and fuchsin, the former of which increases the H. I. C. to pH 7.8, making this the final reaction of our Endo's agar as prepared at that time. We are of the opinion that the reactions for Endo's agar given in some of the replies to our letter of inquiry were taken before the addition of the chemicals instead of afterwards or as ready for use.

We have used the color standards prepared by Hynson, Westcott and Dunning and some made by ourselves according to Medalia's directions, using P. R. as the indicator in both cases. We prefer to use the latter standards as the colors of the unknown and of the standards match closer. We have observed, however, that we have to use 10 cc. of the undiluted Endo's agar instead of 2 cc. diluted with 8 cc. of distilled water in order to check the pH values obtained by comparison with the standards put up by Hynson, Westcott and Dunning. Table 8 illustrates this point.

TABLE 8

Hydrogen-ion concentrations of dilvted and undiluted Endo's agar, expressed in pH. Indicator, phenol red

BATCH NUMBER	BEFORE ADDITION OF CHEMICALS		AFTER ADDITION OF CHEMICALS	
	2 cc. agar plus 8 cc. water	10 cc. of agar	2 cc. agar plus 8 cc. water	10 cc. of agar
1	7.4	7.7	7.9	8.2
2	7.2	7.5	7.7	8.0

We have also noted that the pH value of warm Endo's agar averages 0.2 less than that of the same agar cooled to room temperature so we make it a practice always to cool this agar as well as nutrient agar before taking the H. I. C.

In connection with Endo's agar we have been advised to prepare it according to the Robinson and Rettger (1916) modification, which consists essentially in the use of anhydrous sodium bisulphite in place of sodium sulphite, but we have been unsuccessful in making up a batch which is practically colorless when cold or even light pink. We believe that our trouble is due to the poor quality of sodium bisulphite because no trouble is experienced in other laboratories in preparing Endo's agar according to this modification. When sodium bisulphite is used instead of sodium sulphite the agar must be adjusted to a different reaction prior to the addition of the chemicals because the former will cause a reduction in the H. I. C. and if a final reaction of pH 8.0 is desired this must be taken into account.

We have observed that Endo's agar stored in a flask in the same refrigerator with lactose broth and nutrient agar for a period of seven days did not change in H. I. C. Ordinarily we make 1 liter of this medium every eight days and pour enough plates to last for three or four days, the latter being stored in the refrigerator also. The agar in plates three days old was found to have the same reaction as when poured, i.e., pH 8.0.

We suggest the abandonment of the adjustment of this medium to a certain reaction to P., either that specified in the *Standard Methods* (1920) or in use in any laboratory and the substitution of a H. I. C. of 8.0 to 8.2 for the final reaction of the agar, i.e., as ready for use in the plates.

EOSINE-METHYLENE BLUE AGAR

Composition; grams: distilled water, 1000; Bacto-agar, 15; Bacto-peptone, 10; K_2HPO_4, 2.

A batch of this agar was prepared according to the method given by Levine (1918) and divided into two portions. The two portions were sterilized in the autoclave used for the lactose broth and nutrient agar at 17 lbs. pressure for 20 minutes after this pressure was reached. The reaction of one portion was left unadjusted and was found to have a final H. I. C. of pH 7.1 while the final reaction of the other portion was adjusted to pH 8.0 to agree with that of Endo's agar.

To 250 cc. of each portion, the following amounts of chemicals were added immediately prior to the preparation of the plates:

5 cc. of a 2 per cent aqueous solution of yellow eosine.
5 cc. of a 0.5 per cent aqueous solution of methylene blue.
2.5 grams of Bacto-lactose.

Plates of the two portions of agar were prepared from tubes of lactose broth inocultated with human faeces and different waters and compared after 24 hours incubation at at 37.5°C., in order to see which portion produced the most distinctive appearing colonies. The colonies which developed on the unadjusted agar (pH 7.1) were so much superior to those which developed on the agar adjusted to pH 8.0 that the three observers who judged the colonies had no difficulty in agreeing on the proper H. I. C. of this medium.

The addition of the eosine and methylene blue to this agar makes color so dark that it is difficult to obtain a proper comparison with the standards if the indicator, either B. T. B. or P. R., is added to 10 cc. of the medium. If 2 cc. of the agar, to which the dyes have been added, are diluted with 8 cc. of water and B. T. B. added, the resulting color may be compared without difficulty with standards prepared after Medalia. As the eosine and methylene blue do not alter the H. I. C. of this medium we have decided to take the final reaction on the sterilized agar prior to the addition of these chemicals. If this is done either the color standards put up by Hynson, Westcott and Dunning or those prepared after Medalia may be used as described under nutrient agar.

We have not tried the preparation of this agar with peptones other than Bacto-peptone so we can not be certain that the unadjusted reaction of this medium, prepared with some other peptone, would fall between pH 7.0 to 7.2. Neither have we done any work to ascertain how far below pH 7.0 the reaction could drop without affecting the development of the colonies. As prepared with Bacto-peptone and Baker's analyzed dibasic potassium phosphate we obtain an agar with a final reaction of pH 7.0 to 7.2 on which the various types of colonies of B. coli and B. aerogenes described by Levine (1921) develop nicely.

Endo's agar and the eosine-methylene blue agar for use in the Mt. Hope and Agua Clara laboratories are prepared in the Miraflores laboratory and shipped to the other two in flasks, 250 cc. per flask. The chemicals necessary for the development of the colonies are added at each laboratory when the plates are poured. When gas develops only occasionally in the fermentation tubes, poured plates of each kind of agar, ready for use, are shipped instead of the flasks.

A permanent record of the composition and reaction of each batch of Endo's agar and eosine-methylene blue agar is kept in a note book similar to that used for nutrient agar and lactose broth.

We suggest a range of pH 7.0 to 7.2 for eosine-methylene blue agar for confirming the presence of members of the B. coli group and that the reaction reported be that taken after sterilization, but prior to the addition of the eosine and methylene blue.

REACTION OF PEPTONE MEDIUM FOR THE METHYL RED TEST

This medium is prepared in the Miraflores laboratory according to the formula marked (a) on page 107 of the *Standard Methods* (1920). Proteose-Peptone, Difco, Baker's analyzed dibasic potassium phosphate, and Baker's dextrose are used as the ingredients. As this formula calls for no adjustment of the reaction we have taken the H. I. C. of this medium only recently and found it to average pH 7.4 as ready for use. The substitution of Witte's peptone for Proteose peptone, Difco, gives a medium with an H. I. C. of pH 7.5. We have not accumulated sufficient data on the H.I.C. of this medium to discuss it at greater length.

REACTION OF LITMUS-LACTOSE-AGAR

We abandoned the use of this medium sometime ago but we believe that the final reaction should range between pH 6.8 to 7.2, the same as for nutrient agar and lactose broth. Sörensen gives the pH range of azolitmin as 4.5 to 8.3.

The dehydrated litmus-lactose-agar prepared by the Digestive Ferments Company is stated to be adjusted to a neutral reaction and to have a pH value of 7.0 if sterilized 20 minutes at 15 pounds pressure.

COLOR STANDARDS

1. Standards prepared by The Hynson, Westcott and Dunning Pharmaceutical Laboratory, Baltimore, Maryland

Sets of phenol red color standards prepared by this laboratory[14] were used in the Miraflores, Mt. Hope and Agua Clara laboratories until the latter part of 1920 when an article by Medalia (1920) on "'Color Standards' for the colorimetric measurement of H. I. C. pH 1.2 to pH 9.8" was published. The sets contain 15 color standards in sealed glass tubes, 8.2 cm. long and 1 cm. in diameter, which starting with a minimum pH value of 6.6 advance by increments of 0.2 and 0.1 up to a maximum pH value of 8.6, with the value of each standard marked on the glass. The standards are neatly arranged in a long narrow wooden block which slips into a polished case—dimensions, 3.7 by 11 by 28.5 cm.—for protection against light and injury when they are not in use. According to a reprint of an article by Levy, Rowntree and Marriott (1915) sent out with the sets, the standards are prepared according to Sörensen's (1909) directions from 1/15M solutions of acid potassium phosphate and alkaline sodium phosphate which are mixed in the proportions indicated in Table 9 to obtain the desired pH. Five drops of an aqueous 0.01 per cent solution of phenol red are added to the contents of each tube.

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[14] Similar sets of color standards in 10 cc. ampoule sets, covering any portion of the range pH 1.0 to 10.0, are advertised by the LaMotte Chemical Products Company, 13 W. Saratoga Street, Baltimore, Maryland, U. S. A.

An alcoholic solution of P. R. (phenolsulphonephthalein) 0.01 per cent, supplied with the sets, was first used as an indicator but as it was soon found that the strength of this solution did not remain constant an aqueous solution of the same strength was substituted. The latter solution is kept in an amber glass bottle the rubber stopper of which carries a 1.0 cc. pipette, the upper end of which is protected from dust by a small inverted test tube carrying a wad of cotton. This solution is prepared in liter batches by adding 0.1 gram to about 950 cc. of distilled water in a glass stoppered liter flask. The pH of the distilled water should be taken and if not close to 6.4 the latter should be boiled and cooled before the indicator is dissolved in it. As the indicator dissolves slowly the flask is allowed to stand

TABLE 9

Composition of standards in sets furnished by The Hynson, Westcott and Dunning Pharmaceutical Laboratory

pH	ACID POTASSIUM PHOSPHATE	ALKALINE SODIUM PHOSPHATE
	cc.	*cc.*
6.6	63	37
6.8	51	49
7.0	37	63
7.1	32	68
7.2	27	73
7.3	23	77
7.4	19	81
7.5	15.8	84.2
7.6	13.2	86.8
7.7	11.0	89.0
7.8	8.8	91.2
8.0	5.6	94.4
8.2	3.2	96.8
8.4	2.0	98.0
8.6	Not given	Not given

one or two days with occasional shakings before the solution is made up to the mark. It is then transferred to a brown bottle without filtering. One liter lasts about 6 months among the three laboratories at the end of which time no change is observed in the indicator on comparison with a new solution.

The comparator block, containing one row of three holes, which was furnished with each set, was found to be unsatisfactory and a new block was made with two parallel rows of three holes each and the necessary slits which was based on one described by Hurwitz, Meyer and Ostenberg (1916). Clark (1917) stated: "The device is optically very imperfect but works fairly well." A piece of ground glass was inserted in the back of the block. The outside of the block as well as the slits should be painted with a black enamel.

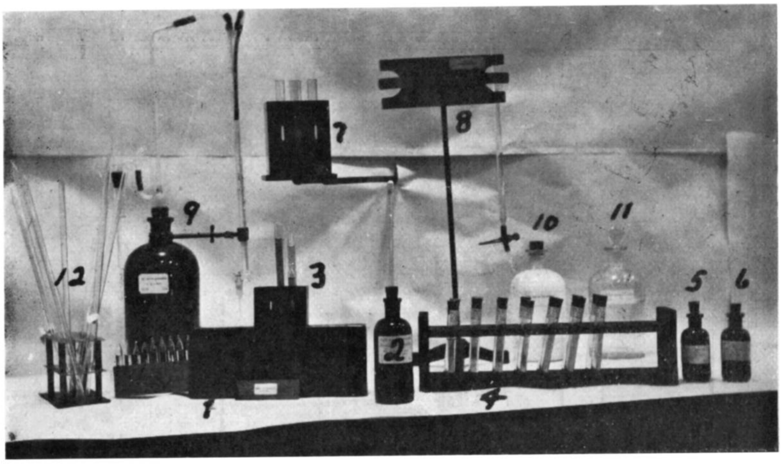

Fig. 2. Apparatus in use in Bacteriological Laboratory of the Miraflores Purification Plant, Canal Zone

1. Set of standards; Hynson, Westcott & Dunning Pharmaceutical Laboratory. *2.* Phenol red indicator, 0.01 per cent. *3.* Comparator block for above set. *4.* Brom thymol blue standards prepared after Medalia. *5.* Brom thymol blue indicator, 0.02 per cent. *6.* Methyl red indicator, 0.02 per cent. *7.* Comparator block for Medalia's standards. *8.* Burette stand, N/20 NaOH. *9.* N/1 NaOH. *10.* Stock solution, N/20 NaOH. *11.* 0.1 per cent HCl. *12.* Pipettes for indicators and media.

The first blocks were made with round holes instead of slits but it was soon found that the latter were superior to the former in making color comparisons. There is a difference of opinion about the shape of the holes through which the colors of the unknown and the standards are compared. The blocks provided with the sets were made up with slits. Medalia (1920) uses slits in his block. Clark (1920) shows round holes in the block illustrated in figure 7 of his book. Cox and Wood (1920) use round holes of two thirds the

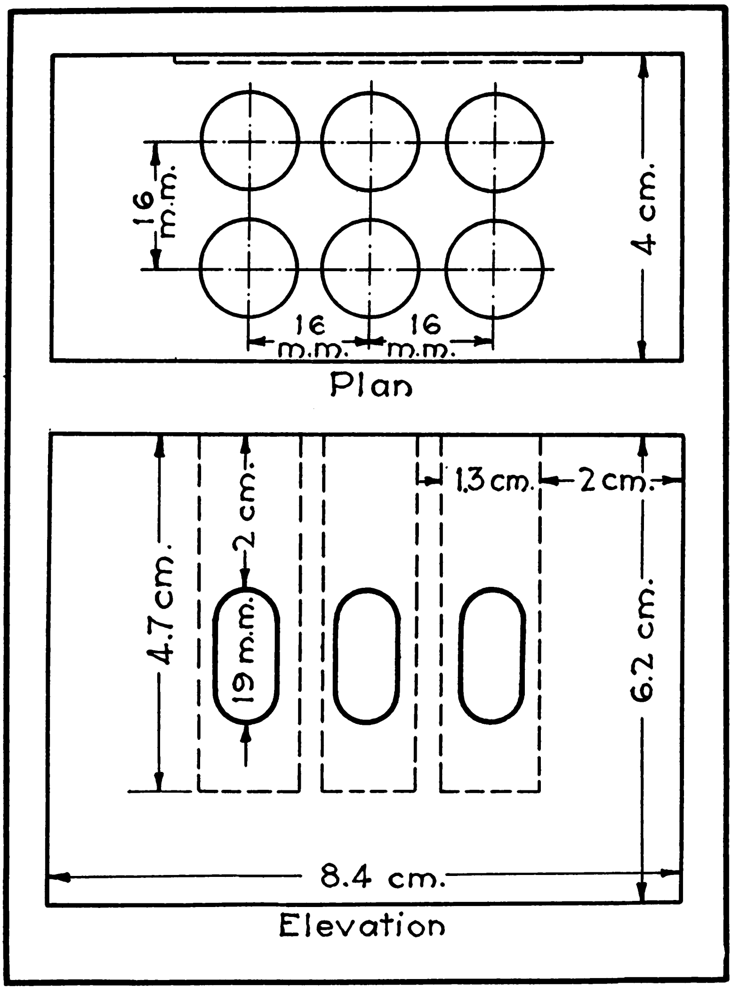

FIG. 3. COMPARATOR FOR USE WITH SET FURNISHED BY HYNSON, WESTCOTT AND DUNNING

size of those in which the test tubes are set. Six men who have worked in the Miraflores laboratory at different times have preferred to use blocks provided with slits. The details of the block which we use may be found in figure 3.

Two test tubes for holding the unknowns are furnished with each set. It is essential that the test tubes in which the medium is placed be of clear colorless glass and of the same diameter as those in which the standards are sealed. The tubes furnished by the manufacturers at the present time or at least up to November, 1920, were unsatisfactory as their diameter was smaller than that of the tubes containing the standards. As a result of this indifference or carelessness of the manufacturers the buyers of these sets must purchase other test tubes from laboratory supply houses, which not only increases the expense and causes delay but is aggravating. It would be a simple matter for the manufacturers to include six test tubes of the same diameter and of the same glass as those in which the standards are sealed. The cost of the complete (standards, indicator and tubes) set as furnished by the manufacturers was $8.50 in November, 1920.

The manufacturers state that the glass tubing in which the standards are sealed is not soluble but we have found that flakes of silica appear in the standards at the end of a few months. However they do not appear to interfere with the color comparison. In regard to the stability of the standards the manufactuers state that: "The stability of the colors is believed to be reasonably permanent, but, if there is evidence of fading, the apparatus should be returned for examination and adjustment after six months." Three of the standards in the Miraflores set (25 months old) are cloudy, a private set (6 months old) contains one cloudy standard, while in two other sets (12 months old) all of the standards have remained clear. In all the standards of each set there is a small amount of coarse flocculent material which remains on the bottoms of the tubes if they are not inverted.

The standards of one of the 12 months old sets and of the 25 months old set were compared against those of the 6 months old set through distilled water with the results shown in table 10.

In one case the difference between the standards ran as high as 0.2 but in the other comparisons the difference was always less than 0.1 and might fairly be set at 0.05. Out of 15 standards in each set, 9 of the 12 months old set matched the 9 similar standards of the 6 months old set. Five of the remaining six standards of the former set were stronger than the similar five of the latter set. Eleven of the standards in the 25 months old set were weaker than the similar standards in the other two sets but in only one case, pH 7.4, did the difference equal pH 0.1. Of the other four standards, two matched the similar standards of the other two sets, one was stronger, while the fourth was broken. These sets will be further discussed under Medalia's standards. Based on our experience with the above four sets we can recommend their use, especially in water works laboratories in which a chemist cannot devote much time to laboratory work, and we consider in general that they may be used for one year, if properly taken care of, before being returned to the manufacturers for adjustment.

TABLE 10

Comparison of standards of three sets of different ages

pH	AGE SET		
	6 months	12 months	25 months
6.6		Matches	Weaker
6.8		Matches	Weaker
7.0		Matches	Weaker
7.1		Matches	Weaker
7.2		A shade pinker	Weaker
7.3		Stronger	Weaker
7.4	Color faded	Stronger	Nearly 0.1 lower
7.5		Matches	Matches
7.6		Matches	Matches
7.7		Stronger	Weaker
7.8		Stronger	Weaker
8.0		Matches	Stronger
8.2		Matches	Weaker
8.4	Matches 8.2 of 12 months old set.	Stronger	Broken
8.6		Matches	Weaker

In determining the pH value of a medium, lactose broth for instance, by means of a set of these standards, 10 cc. of the broth are placed in each of three test tubes, of the same diameter as that of the standards, and cooled to room temperature, after which 1 cc. of the aqueous solution of P. R. is added to one tube. The broth and indicator are then thoroughly mixed by placing the thumb over the end of the tube and inverting the latter. The tubes of broth, distilled water and the standards approximating the color of pair no. 2 are then placed in the comparator shown in figure 3 in the positions shown in figure 4. The block is held between the eyes and a well lighted window in such a position that in looking through the slits the color of pair no. 2 may be compared with the colors of pairs nos. 1 and 3. Various standards are tried until the proper one is found to match the color of the broth plus the indicator. At the upper and lower ends of the scale of pH values a color falling between two standards may be read to the nearest 0.05. The quality rather than the quantity of the color of the unknown is to be matched. The tubes of broth, to which none of the indicator has been added, are used with the standards in order to compensate for the color of the broth itself.

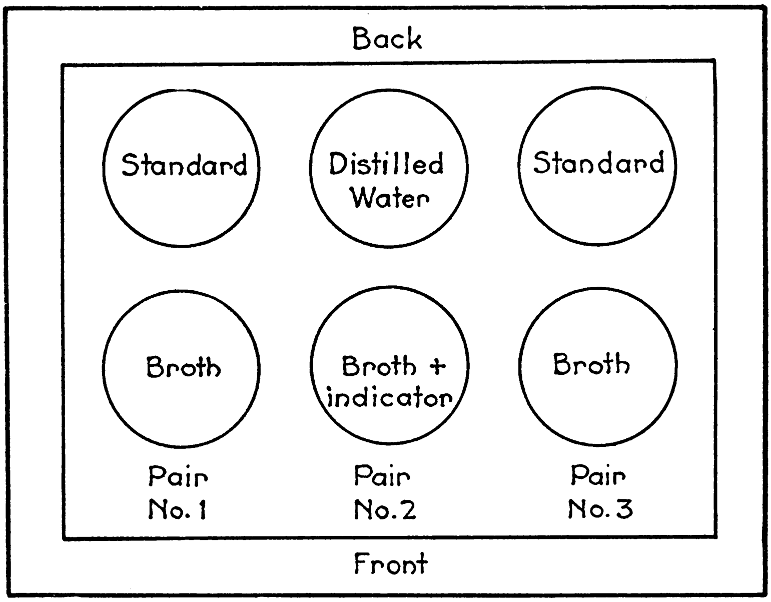

FIG. 4. ARRANGEMENT OF UNKNOWN AND STANDARDS IN COMPARATOR BLOCK FOR DETERMINING H-ION CONCENTRATION

To adjust the reaction of a batch of lactose broth to a pH 7.0, the tubes and standards are set up in the manner just described, the standard pH 7.0 being used in pair no. 1 and the standard pH 7.1 being used in pair no. 3. From a burette N/20 NaOH is run into the tube of broth plus indicator in portions of a few drops at a time, the contents of the tube being mixed after the addition of each portion and the resulting color compared with that of the standards on either side. In this manner there is determined the amount of N/20 NaOH which is necessary to make the color of the broth plus the indicator match the standard marked pH 7.0. Multiply the number of cc. of N/20 NaOH by 5 to obtain the number of cc. of N/1 NaOH to add to 1 liter of broth to adjust the H. I. C. to pH 7.0. In case the medium is too alkaline the titration is made with N/20 HCl in the same manner. Some men prefer to work only with two pairs in the block, omitting either pair no. 1 or no. 3.

After the N/1 NaOH has been added to the batch of broth and thoroughly mixed with it the adjusted reaction is again determined in the same manner. It frequently occurs that the amount of N/1 NaOH theoretically added fails to raise the H. I. C. to pH 7.0 in which case a second addition of the alkali is necessary. The H. I. C. must always be taken after the last addition of the alkali or acid in order to make certain that it is correct before the medium is sterilized.

In the case of lactose broth and nutrient agar, after the amount of N/1 NaOH necessary for the adjustment of the reaction has been determined for one batch, it will be found unneccesary to titrate the succeeding batches with N/20 NaOH as long as the same brands and proportions of the ingredients are used. For example, at the present time we are adding from 40 cc. to 41 cc. of N/1 NaOH to each 4 liter batch of lactose broth to adjust the reaction to pH 7.0 so that it is only rarely that the bacteriologist in charge of the media has to add the alkali in more than two portions, say 40 cc. the first time and 1 cc. the second time, to obtain the proper pH. It frequently happens that the addition of the first large amount will adjust the reaction to the proper pH.

In the first part of his paper Medalia makes the following statement:

> The colorimetric method depends on the color changes that take place in certain indicators at different acid strength or H-ion concentration. The H-ion concentration has been determined electrolytically for a number of solutions ("buffer solutions") containing various chemicals in different dilutions. Such solutions when accurately duplicated are supposed to be of the same H-ion content, and should therefore show similar variations in color, in the indicators used. These solutions are referred to as "standard solutions" of known H. I. C.
>
> The difficulties met with in preparing the "standard solutions" of known H. I. C. in the average bacteriological laboratory constitute one reason why

this method has not come into general use, and furnished the principal stimulus for this research.

On account of lack of space we can only give brief notes about the preparation of the indicators and color standards by Medalia; for details those interested are advised to consult the original paper.

The stock alcoholic solution is prepared by dissolving 0.1 gram of the chosen indicator (powder form) in 50 cc. of 95 per cent ethyl alcohol. It should be stored in a sterilized amber colored bottle with a tight fitting rubber stopper in a dark cup-board. The indicators from which color standards may be prepared, together with the ranges of H. I. C. which they cover are given in table 11.

The watery or aqueous indicator solution is prepared by adding 5 cc. of the stock alcoholic solution by a sterile pipette (10 cc. in the case of phenol red) to 45 cc. of distilled water (40 cc. in the case of phenol red) which has been previously sterilized in an amber colored bottle at 10 pounds pressure for 30 minutes. On removal from the autoclave insert a sterilized rubber stopper and store in the dark.

For the color standards Medalia advises the use of B. B. H. test tubes (thick walled, without lips, 130 by 16 mm. outside diameter). Tubes of non-corrosive glass should be used as the alkaline standards corrode the glass of ordinary tubes. It is of great importance to select tubes of the same diameter and of glass which is clear and of the same shades of color.

In tables 3 to 10 inclusive of his article Medalia gives the detailed composition of the pairs of tubes comprising the 'color standards' of thymol blue in the acid range (pH 1.4–2.6), brom phenol blue (pH 3.4–4.6), methyl red (pH 4.6–5.8), brom cresol purple (pH 5.4–6.6), cresol red (pH 7.4–8.6), thymol blue in the alkaline range (pH 8.2–9.4). The tubes are set up as shown in figure 5 and 10 cc. of HCl are added to one row while 10 cc. of NaOH are added to the other row, except in the thymol blue acid range, 0.0001 of HCl is added to the row of tubes marked N/20 NaOH in figure 5 and 0.5 per cent HCl is added to the other row of tubes. The amounts of each indicator to be added to the tubes are the same as shown in figure 5. To save space the tables in detail will not be reproduced here, but table 11 shows the strength of acid and alkali to be used with each indicator, the strength of the latter and the pH of each pair of tubes.

TABLE 11

pH *values of pairs of color standards prepared from various indicators*

INDICATOR	ACID AND ALKALI	pH OF PAIR NUMBER						
		1	2	3	4	5	6	7
Thymol-blue acid range, 0.02%	0.001% HCl 0.5% HCl	1.4	1.6	1.8	2.0	2.2	2.4	2.6
Brom phenol blue, 0.02%	N/20 NaOH 0.1% HCl	3.4	3.6	3.8	4.0	4.2	4.4	4.6
Methyl red, 0.02%	N/20 NaOH 0.1% HCl	4.6†	4.8	5.0	5.2	5.4	5.6	5.8
Brom cresol purple, 0.02%	N/20 NaOH 0.1% HCl	5.4	5.6	5.8*	6.0	6.2	6.4	6.6
Brom thymol blue, 0.02%	N/20 NaOH 0.1% HCl	6.4	6.6	6.8	7.0	7.2	7.4	7.6
Phenol red, 0.04%	N/20 NaOH 0.1% HCl	7.0	7.2	7.4	7.6	7.8	8.0	8.2
Cresol red, 0.02%	N/20 NaOH 0.1% HCl	7.4	7.6	7.8	8.0	8.2	8.4	8.6
Thymol blue, alkaline range, 0.02%	N/20 NaOH 0.001% HCl	8.2	8.4	8.6	8.8	9.0	9.2	9.4

* "According to phthalate and phosphate NaOH mixtures, pairs 3, 4, 5, 6, 7, should read: pH 5.9; 6.1; 6.3; 6.5; 6.7."

† "There was a very slight difference between the phthalate NaOH mixture and the 'standard colors' in the first three pairs: Pair no. 1 = pH 4.7; pair no. 2 = pH 4.9; pair no. 3 = pH 5.1 according to the phthalate NaOH mixture, the rest were the same in both."

The following directions for the titration of culture media are reprinted from Medalia's article.

Titration of culture media

For the titration of a culture medium[15] three test tubes, similar to those used for the "color standards," are filled, each with 2 cc. of the filtered medium

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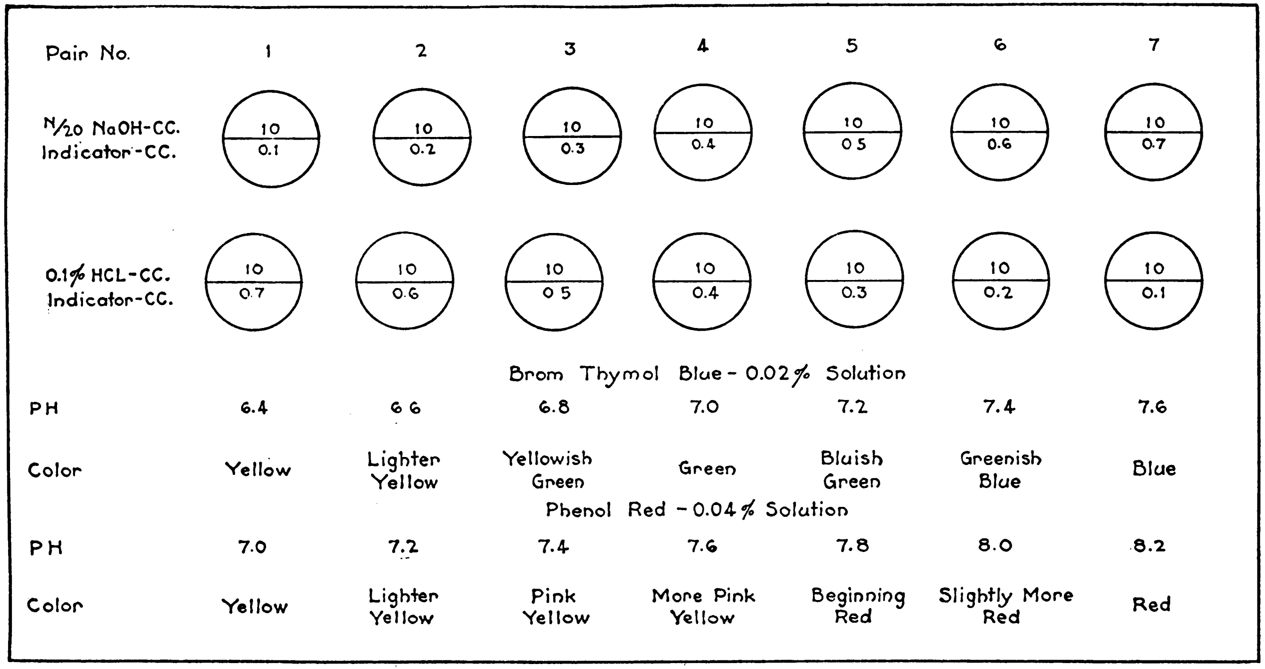
[15] Hot fluid agar or gelatin media, before final titration, can be titrated as described. The 2 cc. of the agar or gelatin when diluted with 8 cc. of water, remain fluid for a much longer time than is necessary to carry out the titration.



Pair No.	1	2	3	4	5	6	7
N/20 NaOH-CC. Indicator-CC.	10 0.1	10 0.2	10 0.3	10 0.4	10 0 5	10 0.6	10 0.7
0.1% HCL-CC. Indicator-CC.	10 0.7	10 0.6	10 0 5	10 0.4	10 0.3	10 0.2	10 0.1
			Brom Thymol Blue - 0.02% Solution				
PH	6.4	6 6	6.8	7.0	7.2	7.4	7.6
Color	Yellow	Lighter Yellow	Yellowish Green	Green	Bluish Green	Greenish Blue	Blue
			Phenol Red - 0.04% Solution				
PH	7.0	7.2	7.4	7.6	7.8	8.0	8.2
Color	Yellow	Lighter Yellow	Pink Yellow	More Pink Yellow	Beginning Red	Slightly More Red	Red

FIG. 5. COMPOSITION OF "STANDARD COLORS" PREPARED WITH BROM THYMOL BLUE AND WITH PHENOL RED

and 8 cc. of distilled water. To one of these 0.8 cc. of the 0.02 per cent of the brom thymol blue indicator is added and compared with the "color standards" tubes. The other two tubes are used to offset the color of the medium in the "comparator" block described below. The fluid to be titrated is placed in the center row of the "comparator" block having two test tubes of distilled water behind it. On either side are placed the two pairs of test tubes nearest to the pH desired (pairs 4 and 5 pH 7 and pH 7.2). In front of each pair is placed the tube containing 2 cc. of the medium and 8 cc. of distilled water to offset the color of the medium. It is absolutely necessary to compensate for the color of the medium in this way or the result will be misleading. N/20 NaOH is carefully run in the tube that is being titrated until it matches pair No. 4 = pH 7. Twenty-five times the amount of N/20 NaOH used, will represent the amount of normal NaOH necessary to neutralize 1 liter of the medium.

$$\frac{1000 \text{ cc.}}{2 \text{ cc.}} \times \frac{1}{20} = 25$$

If the medium is too alkaline add N/20 HCl until the color matches and calculate the amount of N/1 HCl to be added to one liter of medium in the same way. The same factor is used whatever the pH value selected for the reaction of the medium. The medium should be retitrated after the addition of the alkali or acid as the case may be. The use of the "comparator block" such as described by Hurwitz, Meyer and Östenberg (1916) modified to suit our purpose, was found indispensable.

The block is made by boring three rows of three holes in each row. The holes are 2 cm. in diameter and 9 cm. deep. The block measures 13 cm. high by 9 by 10 cm. Each row of holes is connected by a slit that goes all the way through, 2.5 cm. high by 1.25 cm. wide, the upper edge of the slit being 4 cm. from top of block. The block is painted with black enamel paint including the slits. It is best not to paint the holes that hold the test tubes.

Up to the time of writing this paper, P. R., B. T. B. and M.R were the only indicators available for our use. We now have all of the eight indicators listed by Clark and Lubs. These indicators may be purchased either from the LaMotte Chemical Products Company, 13 W. Saratoga Street, Baltimore, Md., Hynson, Westcott and Dunning in the same city; or Grahame Chemical Co., Rochester, N. Y. At the present time 10 grams of either brom thymol blue, thymol blue, cresol red, brom cresol purple are worth about $18.00.

In preparing solutions of B. T. B., P. R. and M. R. both alcoholic and watery, we have followed exactly the directions given by Medalia. We have experienced trouble with both kinds of solutions spoiling and thereby giving erroneous results but the watery solutions have spoiled more frequently than the stock alcoholic solutions. A stock solution of P. R. kept in a brown bottle in a closet spoiled at the end of 45 days so that a watery solution made from it was altogether too light in color. A stock solution of B. T. B. made up at the same time is still in good condition. During the first few weeks of our work the "indicator watery solutions" spoiled in a few days. The 50 cc. of the watery solution of B. T. B. remain unchanged during the 20 days it lasted. We intend to insert 1 cc. pipettes in the rubber stoppers of the bottles and cover the upper ends by small inverted test tubes carrying a wad of cotton to prevent the entrance of dust. While Medalia does not recommend this procedure we believe that it is preferable to the insertion of a non-sterile pipette into the indicator and it is not very practical to always keep a supply of graduated sterile pipettes on hand. At the present time we are not satisfied with the "indicator watery solutions" and intend to to do some work on this feature which seems to be the weakest point in Medalia's methods.

In preparing the color standards we used test tubes, 125 mm. long and 14 mm. inside diameter, without lips and of medium thickness. The tubes were washed only in warm water to avoid the formation of a coating on the glass. We consumed a considerable amount of time in selecting the necessary number of tubes made from glass of the same shade of color but it is essential to do this as otherwise erroneous results will be obtained later on.

In tables 3 and 4 of his paper Medalia gives the composition of each pair of the seven pairs of standards prepared with B. T. B. and P. R., "the color change of each, as nearly as can be described, and the pH value of each pair." We have arranged the data in these tables in diagramatic form in Fig. 5, in which the circles represent the test tubes as set up in a rack and the figures inside show the respective amounts of N/20 NaOH or 0.1 per cent HCl and the watery solution of the indicator to be added to each tube. This diagram will serve as a guide for the preparation of color standards with any of the indicators listed in table 11 of this paper which shows the strengths of the HCl and NaOH used with the different indicators. By following the directions given in the quotation from Medalia's paper under the heading of "Preparation of 'color standards'" in connection with our figure 5 no trouble should be experienced in preparing any of the sets of color standards included in table 11. The N/20 NaOH was prepared from Baker's Analyzed NaOH purified by alcohol without attempting to remove the carbonates, and its strength was not determined by titration against an acid solution of known value. Sterile distilled water was used however. The 0.1 per cent HCl was prepared from ordinary "C. P." concentrated acid without redistilling it.

The details of the comparator are shown in figure 6. As in the case of the other comparator shown in figure 3 a piece of ground glass was inserted on the back side. No trouble was experienced

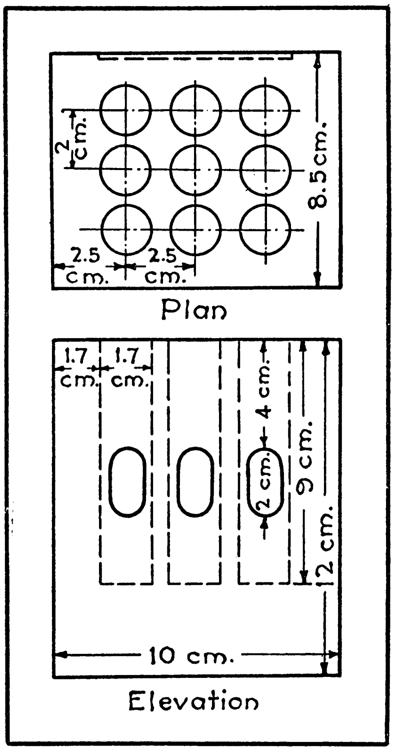


FIG. 6. COMPARATOR FOR USE WITH STANDARDS PREPARED AFTER MEDALIA

in drilling the vertical holes close together on a drill press. In the Miraflores laboratory the comparator was mounted on a swinging bracket fastened to the window frame over the bench so that it is unnecessary for a man to hold it while comparing colors and changing the standards. In determining the H. I. C. of lactose broth or any other medium the tubes of broth and the pairs of color standards are set up as illustrated in figure 7. The color standards must always be used in pairs of acid and alkali as set up in figure 5, each pair containing a total of 0.8 cc. of indicator. While the pH value of each pair differs by pH 0.2 the readings may be interpolated to pH 0.1. Clark (1920) says:

> Certain general considerations have led us to believe that for most work estimation of pH values to the nearest 0.1 division is sufficiently precise,and that this precision can be obtained when the composition of the medium permits if the comparison standards differ by increments of 0.2 pH.

For standardizing the color standards prepared with brom thymol blue and phenol red we used solutions of sodium hydrate and monobasic potassium phosphate prepared according to the directions given on pages 70–76 of Clark's book (1920).

> The water used in the crystalization of the salts and in the preparation of the stock solutions and mixtures should be redistilled. So-called "conductivity water," which is distilled first from acid chromate solution and again from barium hydroxid, is recommended, but it is not necessary.

The water which we used was distilled from the still mentioned under lactose broth and immediately before use was reboiled and cooled. The H. I. C. of the water as used was pH 6.8.

Standard buffer solutions are advertised by the LaMotte Chemical Products Company, and by the Grahame Chemical Company 100 Rockingham Street, Rochester, N. Y. Meacham, Hopfield, and Acree (1920) give the following footnote concerning the latter company:

> The Grahame Chemical Company now sells a sterile single buffer solution which remains constant indefinitely and covers all pH values from 1 to 14 very accurately and replaces the 5 or 6 buffer solutions employed heretofore. They also furnish sterile buffer tablets, with or without admixed standardized quantities of different indicators, which cover all pH values from 1 to 14 in steps of 0.2 pH. These sterile tablets are added to sterile culture media or to water to give the desired pH values, and have been found very useful in saving time and securing accurate results.

We have not used the products of either of the above companies so we can neither criticise nor recommend their products, but they can undoubtedly give references to reputable parties who have used their products.

In the *Standard Methods for the Bacteriological Examination of Milk,*" (1921) the following instructions and comments are given under the heading, "Reaction."

Each batch of finished medium should be tested before use as to its final reaction after sterilization. This test is made as follows: Put 4 cc. of distilled water at 30 to 40°C. (not warmer) in a test tube. Add 1 cc. of the agar to be tested and then 10 drops of the indicator, brom thymol blue 1 (0.04 per cent solution in 95 per cent alcohol). The resulting color should be either a yellowish green or vary to a deeper shade of grass green. To one whose eye is trained this shade of color is sufficient.

These shades may be accurately determined by means of the buffered solutions of Sorensen[16] or of Clark and Lubs.[17]

However, they may be approximately determined by comparing the tube of agar containing the indicator with a set of color tubes after the method of Barnett and Chapman.[18]

Select 12 test tubes of even caliber and place in two rows of 6 each. In each tube of one row put 5 cc. of a dilute alkali (as, for example, twentieth normal sodium hydroxide). In each tube of the other row put 5 cc. of very dilute acid (one drop of concentrated sulphuric or hydrochloric to 100 cc. of distilled water is sufficient). Avoid stronger acid.

Add indicator to the tubes as follows:

Acid tubes	Alkali tubes	H-ion concentration
9 drops	1 drop	pH 6.2
8 drops	2 drops	pH 6.4
7 drops	3 drops	pH 6.7
6 drops	4 drops	pH 6.9
5 drops	5 drops	pH 7.1
4 drops	6 drops	pH 7.3

The tubes are to be viewed in pairs of acid and alkali, each pair containing the sum of ten drops of indicator.

If preferred, double these quantities may be used throughout and the indicator measured in fractions of a cubic centimeter instead of drops. That is, 2 cc. of agar should be taken for testing. This should be added to 8 cc. of distilled water. 1 cc. of indicator should be used. In comparing with the Barnett and Chapman tubes, use 10 cc. of dilute acid or alkali in each tube, and add the indicator in tenths of a cubic centimeter instead of in drops.

All of the test tubes used in this determination must be of the same diameter and of clear glass.

The pH values corresponding to the color pairs (acid and alkali) prepared by the method of Barnett and Chapman have been worked out by Medalia.

In reference to the first three quoted paragraphs we do not see the sense of making a qualitative determination of the H. I. C. as di-

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[16] Sörensen, S. P. L. Ztschr. Biochem., 21, 131, 201, 1909; Ergebn. Physiol., 12, 393, 1912.

[17] Clark, W. M. and Lubs, H. A. The colorimetric determination of hydrogenion concentration and its application in bacteriology. Jour. Bact., 2, 1–34, 109–136, 191–236, 1917.

[18] Barnett, G. D. and Chapman, H. S. Colorimetric determination of reaction of bacteriologic mediums and other fluids. Jour. Amer. Med. Ass., 70, 1062–1063, 1918.

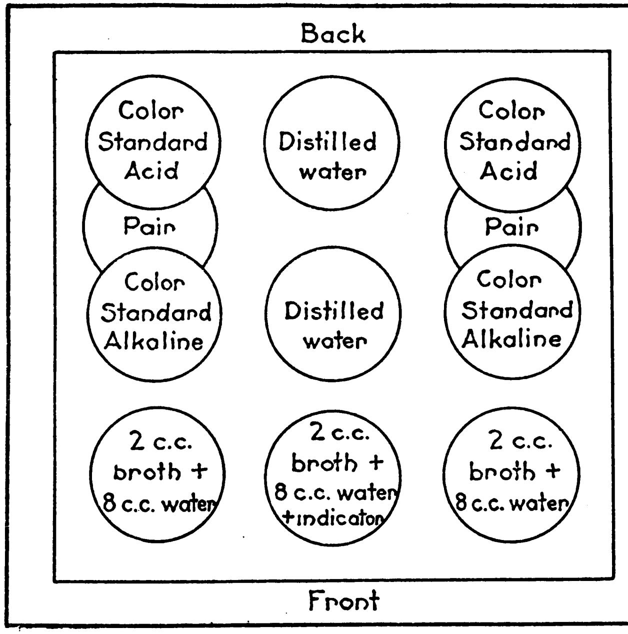

FIG. 7

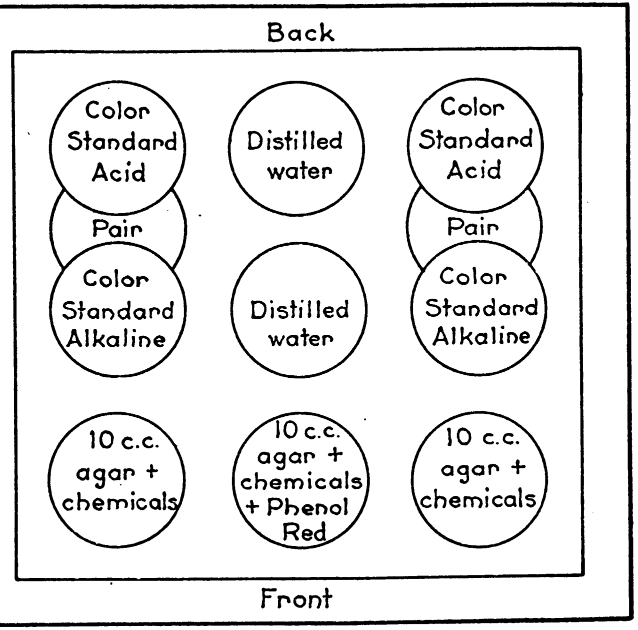

FIG. 8

FIG. 7. ARRANGEMENT OF BROTH AND STANDARDS IN MEDALIA'S COMPARATOR
FIG. 8. ARRANGEMENT OF ENDO'S AGAR AND STANDARDS IN MEDALIA'S COMPARATOR

rected in the first paragraph and checking the shades of color by buffered solutions made according to the directions of Sörensen or of Clark and Lubs. If the time is taken to make the latter solutions, and considerable time is taken in the preparation of the chemicals, why not finish the job and make up a set or sets of color standards according to Medalia to go with these accurately prepared solutions of known H. I. C.? If only a qualitative determination of the H. I. C. of a medium is to be made why not check the shades of color by means of the color chart in Clark's book (1920)?

Inasmuch as the work of Medalia (1920) is referred to in the last quoted sentence and we presume, therefore, although we are not certain that his paper was available at the time these *Standard Methods* were prepared, we are of the opinion that the space given to the preparation of color tubes after Barnett and Chapman should have been given preferably to a brief description of the preparation of Medalia's color standards. Perhaps more space would have been required for the description but a slight increase in the cost ($0.30) of these *Standard Methods* would not have put them out of anybody's reach and as far as accuracy is concerned there is no comparison between the color standards of Medalia and of Barnett and Chapman because those of the former are easily superior. In addition to their being more accurate, there is nothing difficult in their preparation and they are sufficiently permanent to compensate for the increased time required for their preparation. In view of the increasing use of color standards for determining the H. I. C. of media we think that more space should have been given to this subject in the *Standard Methods for the Bacteriological Examination of Milk*, because it is of importance to men in both water and milk laboratories and on account of being published later than the *Standard Methods of Water of Water Analysis* (1920) the opportunity was presented for a more complete description of the subject than was possible in the latter book.

According to Cox and Wood (1921) sets of phenol red color standards have been in use in the laboratories of the Army Medical School and have been sent out to the other larger Army laboratories for use:

> The use of these indicators and standard buffer mixtures in the adjustment of the reaction of bacteriological media was begun in the summer of 1918 at this institution,[19] and has been continued with increasing satisfaction and

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[19] Division of Laboratories, Army Medical School, Washington, D. C.

confidence. The question of more or less permanent standards arose, and was met first by the use of standard solutions, with indicator added, in test tubes closed with corks wrapped in tin foil. These solutions were found to hold their colors very well for a number of weeks, when carefully protected from the light when not in actual use, and kept tightly corked. The sealing of these solutions in ampoules was next tried, and found to be very satisfactory, if the ampoules were of the best quality glass. Phenol red sets were sent out to the larger Army laboratories at three or four months intervals, and the use of this method of adjustment of the reaction of media was urged. On renewal, the return of the old sets was requested, and their colors compared with those of fresh standards. At first, these comparisons showed marked changes in the old sets, the tubes above 7.6 as a rule showing the intensest possible color with phenol red, showing a reaction at least as alkaline as 8.4. (The phenol red range is extensive enough for media adjustment for routine work with the ordinary pathogenic bacteria.) As the quality of the glass of the old sets improved, the changes became less marked, and at present the standard sets distributed from the Army Medical School are test tubes of Pyrex or Non-sol glass, drawn out in the upper third before the addition of the solutions, and then sealed in a blast. We hope that these standards will prove to hold their colors very truly for at least five or six months.

We have made a considerable number of comparisons of the H. I. C. of lactose broth, nutrient agar, and Endo's agar with the color standards of the sets obtained from The Hynson, Westcott and Dunning Pharmaceutical Laboratory and those which we have prepared according to Medalia's directions. In Tables 12 and 13 there will be found the results of some of the comparisons.

From the above table we see that four observers, working independently with three Hynson, Westcott and Dunning sets of different ages, obtained H-ion concentrations on the same batch of nutrient agar which differed only by pH 0.1, the minimum reading being pH 7.1 and the maximum pH 7.2. The 6 months and the 12 months old sets gave the same readings. The same observers, using the same nutrient agar and Medalia's color standards prepared with B. T. B. differed only by pH 0.1, the minimum reading being pH 6.9 and the maximum, pH 7.0, using another set of Medalia's color standards prepared with P. R., the same difference and the same maximum and minimum were obtained. Comparing the readings taken with either set of Medalia's standards with these taken with the 6 months old set of Hynson, Westcott and Dunning, there is found a maximum difference of pH 0.3 and a minimum difference of pH 0.1. For any one observer using the standards of either Hynson, Westcott and Dunning (6 months old) or `of

Medalia the maximum difference in the H. I. C. of the agar was pH 0.2. Each of the four observers obtained a lower H. I. C. of the nutrient agar with Medalia's B. T. B. color standards than with the 6 months old Hynson, Westcott and Dunning set of color standards.

TABLE 12

Hydrogen-ion concentrations of nutrient agar after sterilization, expressed in pH

OBSERVER	STANDARDS FROM HYNSON, WESTCOTT AND DUNNING			MEDALIA'S STANDARDS	
	6 months old	12 months old	25 months old	Brom thymol blue	Phenol red
A	7.1	7.1	7.2	6.9	6.9
B	7.1	7.1	7.2	6.9	6.9
C	7.1	7.1	7.1	7.0	7.0
D	7.2	7.2	7.2	7.0	6.9

TABLE 13

Hydrogen-ion concentrations of two batches of lactose broth after adjustment of reactions, expressed in pH

OBSERVER	STANDARDS FROM HYNSON, WESTCOTT AND DUNNING			MEDALIA'S STANDARDS	
	6 months old	12 months old	25 months old	Brom thymol blue	Phenol red
First batch					
A	7.3	7.3	7.4	7.0	7.0
B	7.3	7.3	7.4	7.0	7.0
C	7.3	7.2	7.4	7.0	7.0
D	7.3	7.3	7.3	7.0	7.0
Second batch					
A	8.6*	8.6*	8.6*		8.1
B	8.6*	8.6*	8.6*		8.2
C	8.6*	8.6*	8.6*		8.2
D	8.6*	8.6*	8.6*		8.1

* The hydrogen-ion concentration of the broth was greater than 8.6 but this was the highest standard in the sets.

From the above table we see that four observers, working independently with the same three sets of Hynson, Westcott and Dunning standards, obtained H-ion concentrations on the "first batch" of lactose broth which differed only by pH 0.2, the minimum reading being pH 7.2 and the maximum, pH 7.4. The 6 months and the 12 months old sets gave the same readings with one exception. The same observers, using the same lactose broth and Medalia's color standards, prepared with B. T. B., agreed on the readings, pH 7.0 being obtained by each of the four. The same was the case with the P. R. color standards. Comparing the readings taken with either set of Medalia's standards with those taken with the 6 months old set of Hynson, Westcott and Dunning there is found a maximum difference of pH 0.4 and a minimum difference of pH 0.2. In all cases a lower H. I. C. was obtained with Medalia's color standards than with the 6 months old set of Hynson, Westcott and Dunning.

The "second batch" of lactose broth in the same table had been adjusted to a neutral reaction with phenolphthalein. The four observers found that the color produced with phenol red was greater than that of the highest standard in the Hynson, Westcott and Dunning sets, pH 8.6. Using Medalia's P. R. standards two of the observers obtained a H. I. C. of pH 8.1 while the other two obtained pH 8.2. In the case of this broth the H. I. C. obtained with the Hynson, Westcott and Dunning sets was clearly way off because the shades of color of the broth plus the P. R. and of the standards were entirely different, so much so that no attempt would be made to use these sets except to show that Medalia's standards were greatly superior.

In all comparisons we have found that the shades of color of 2 cc. of the medium plus 8.0 cc. of water plus the indicator and of Medalia's standards (prepared either with B. T. B. or P. R.) matched more closely than did the shades of color of 10 cc. of the medium plus 1 cc. of P. R. and of the Hynson, Westcott and Dunning standard. The superiority of the first method of determining the H. I. C. is due partly to the greater brilliancy of the standards and indicator, partly to the effects of the dilution, partly to the use of tubes of larger diameter, and partly to the use of the triple sets of tubes in the comparator. Between the B. T. B. and P. R. standards we prefer to use the former when the range of H. I. C. permits because we find the color shades match a little closer. In determining the final reaction of Endo's agar, to which sodium sulphite and fuchsin have been added, we find that the shades of color, developed on the addition of P. R., match very closely. We use the set up shown in figure 8 in this case.

Within the range of pH 6.8 to 7.2, in which the reactions recommended in this paper for lactose broth and nutrient agar will fall, it is our opinion, based on the data in tables 12 and 13 that the H. I. C. of either kind of media may be determined within, a maximum variation of pH 0.3 from the true value. For Endo's agar the final reaction may be taken before the addition of the fuchsin and sodium sulphite at which point the H. I. C. will be about pH 7.0 or 7.2. However at the present time or until such of Medalia's standards are placed on the market we recommend the use of the Hynson, Westcott and Dunning sets of standards or similar sets from other companies in the smaller laboratories because the final reactions of media may be obtained more accurately than by the qualitative method, as we term it, in which the reactions are adjusted by watching for a change in color of the indicator without any comparison with standards.

Whenever it is possible we recommend the use of the color standards prepared according to Medalia's directions because the shades of color of the unknown and the standards match closer and the H. I. C. may be determined more accurately or within a maximum variation of pH 0.1 from the true value, providing of course that the standards have been carefully prepared and checked against solutions of known H. I. C. For routine work, including the preparation of nutrient broth, sugar broths, nutrient agar and gelatine, lactose agar, and Endo's agar and the adjustment of the final reactions within the ranges recommended in this paper, one may get along very nicely with a set of B. T. B. standards with a range of pH 6.4 to 7.6 if the final reaction of Endo's agar is taken at the point mentioned in the preceding paragraph.

SUMMARY AND CONCLUSIONS

We are of the opinion that the reactions of culture media used in water works laboratories should be adjusted by the colorimetric method of determining the H-ion concentration.

We are of the opinion that definite H-ion concentrations should be used for the reactions of nutrient agar, nutrient gelatine, sugar broths, etc., rather than changes in indicators described as "faint pink with phenol red," "distinct grass green" with brom thymol blue, or "the first faint trace of permanent pink to appear with phenol red."

For nutrient agar and gelatine, lactose agar, and nutrient and sugar broths, we suggest that the final reactions, i.e., the reactions of the media as ready for use, be adjusted to come within the range of pH 6.8 to 7.2. For Endo's agar we suggest that the final reaction, after the addition of the fuchsin, sodium sulphite or sodium bisulphite, and lactose, be adjusted to fall within a range of pH 8.0 to 8.2.

In reporting the reaction of a medium, the H. I. C. given should be the final one, i.e., the reaction of the medium as ready for use, unless otherwise stated.

To men in smaller laboratories, with a limited amount of time, we recommend a set of color standards prepared by one of the commercial laboratories.

For more accurate work we recommend the use of color standards prepared according to Medalia's (1920) directions, for we have found them to be superior to those of Barnett and Chapman and those offered for sale by commercial laboratories.

We wish to acknowledge the assistance of Mr. A. H. Khachadoorian who prepared the KH_2PO_4–NaOH mixtures of known H. I. C. and checked various readings with the color standards. Messrs. A. M. Nolte, W. C. Dunn, and W. H. Beers, Jr., have also assisted us as observers. We also wish to thank the directors of various laboratories who kindly answered our letter of inquiry regarding the reactions of culture media.

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